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The activity of telomerase has been shown to be absent in normal somatic cells, with the exception of stem cells. The reactivation of telomerase has been seen as an early event in most cancers, especially breast cancer. I have shown that the inhibition of telomerase, by various types of inhibitors, led to the inhibition of cell growth via telomere-based mechanisms. To understand the mechanism of telomere shortening and growth inhibition, microarray analyses were performed to detect changes in the transcriptional profiling. The effects of telomerase inhibitors were found to be independent of p53 and pRB status. Furthermore, I showed that telomerase inhibitors and tamoxifen could prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells. Microarray analyses detected changes in the transcriptional profiling after treatment with tamoxifen and telomerase inhibitors. Also, Northern analyses of the spontaneously immortalized breast epithelial cells showed that while the cells were negative for estrogen receptor-alpha (ERα) by histology, the data show that the cells possibly contain ERβ. Tamoxifen mechanism's may be to utilize orphan receptors. These studies should lead to new insights in preventing the occurrence or recurrence of breast cancer.

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Introduction

Li-Fraumeni Syndrome (LFS) is characterized by heterozygous mutations in the tumor suppressor gene p53 (1). Alterations in the p53 gene are thought to lead to genomic instability and allow for continued cellular proliferation, leading to further instabilities (2). Studies of the importance of p53, and identification of the LFS germ line mutations of p53, have led to an understanding of the cancer risk to LFS families. Among women in affected LFS families, breast tumors are the most prevalent cancer (afflicting at least 50%), with one quarter of the breast cancers diagnosed before age 30 and 89% diagnosed before age 50 (1,3-4). The molecular mechanism of the specific increased incidence of breast cancer as opposed to other cancers in families affected by LFS is not completely understood (5-6). One of the Li-Fraumeni Syndrome (LFS) cell strains that is used as a model for this project is derived from a patient who had surgery for breast cancer. These human mammary epithelial (HME) LFS cells are telomerase silent, grow in defined medium for approximately 50 population doublings then undergo a crisis stage, reproducibly immortalize and then express telomerase (7).

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the end of chromosomes to provide genomic stability and to provide a source of expendable DNA due to the end replication problem where one DNA strand cannot complete its end during replication (8-10). Telomere length has been shown to decrease with time (and with increasing age). Maintenance of telomeres has been shown to involve telomerase activity, which acts as a reverse transcriptase to add base pairs to the ends of chromosomes (11). It has been proposed that an immortalized cell emerges (a hallmark of most cancer cells) from a stage called crisis when telomerase or another mechanism to maintain telomere stability is activated (12). Once telomerase is activated it stabilizes telomere length and permits continued cell division. Most cancer cells have been shown to contain short but stable telomeres compared to parental cells. Beside germ line and stem cells of renewal tissues, other cells having telomerase activity are cancer cells (90% of those tested) (13).

Telomerase has been shown to become active early in breast cancer progression (13 for review). Thus, the proposed study will determine, using a cell culture model system, if inhibiting telomerase activation has the potential to prevent the occurrence or recurrence of breast cancer. The approach is to examine the effects of telomerase inhibitors, and other putative chemopreventive agents such as tamoxifen, on the growth of immortalized breast epithelial cells and the frequency of spontaneous immortalization in human breast epithelial cell from individuals predisposed to breast cancer.

As telomerase is re-expressed in cancer cells, the critically short telomeres may be favored to be elongated by telomerase. The average telomere length in these cells becomes stable at lengths well below normal cells. These shorter lengths are at a critical length for cell survival. The difference in telomere lengths between normal and cancer cells provides for a mechanism that may specifically target and inhibit the growth of cancer cells. It is proposed that the inhibition of telomerase may lead to the growth inhibition of immortalized human mammary epithelial cells (HMECs) and to the prevention of the spontaneous immortalization of HMECs through telomere shortening.

Body and Conclusions

In the first year, the "Statement of Work's Task 1" was to inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors. I showed that the inhibition of telomerase led to the inhibition of cell growth via telomere-based mechanisms (14, Annual Report 2001, Appendix A1). While non-toxic concentrations of tamoxifen did not inhibit telomerase activity, the 2'-O-meRNA specific to the RNA component of telomerase or the dominant negative hTERT did inhibit telomerase activity. Shortening of telomeres and subsequent growth inhibition followed inhibition of telomerase activity. The method of growth inhibition was determined as apoptotic due to critically shortened telomeres. The next task was to understand the mechanism for the induction of cell death via telomere shortening in these cells. During the second year, microarray analyses were performed to detect any change in the transcriptional profiling after telomerase inhibition. Additional microarray analyses will be performed to verify the preliminary results. Also during the second year, another type of telomerase inhibitor, oligonucleotide phosphoramidates, was shown to be an efficient telomerase inhibitor and potential therapeutic agent (15-16, Appendices A4-5).

In addition to the tasks outlined for the first year, I was able to show that telomerase inhibitors and tamoxifen can prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells, which have not reached cellular crisis, by the prevention of telomerase activation (17, "Statement of Work's Task 2," Annual Reports 2001 and 2002, Appendices A2 and A7). The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors. The finding that treating cells prior to crisis with anti-telomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells *in vitro* indicates a potential approach for the development of rational chemoprevention strategies using both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization offers a new intermediate endpoint for validating novel chemopreventive agents. The next task was to understand the mechanism for preventing the spontaneous immortalization of these cells (Annual Report 2002). During the second year, microarray analyses were performed to detect any change in the transcriptional profiling after tamoxifen treatment. Northern analyses of the spontaneously immortalized LFS-derived breast epithelial cells showed that while the cells were negative for estrogen receptor-alpha (ERα) by histology, the cells show that they possibly contain ERβ. The method of action by tamoxifen may be to utilize orphan receptors.

In the third year, "Statement of Work's Task 3" was to understand the fate of breast epithelial cells with different p53 and pRB status when treated with telomerase inhibitors. Cell growth was monitored and cells were collected for telomerase activity, telomere length, and protein analyses. Apoptosis and senescence assays were performed to determine fate of cells. Data suggest that growth inhibition and apoptosis due to telomerase inhibition is independent of p53 and pRB status. As a side project to understand the role of the p16/Rb pathway in telomere biology and replicative senescence in breast epithelial cells, we showed that inactivation of p16 is not required to immortalize epithelial cells and is a stress response protein (18, Annual Report 2002, Appendix 6). Telomere shortening to a critical length may trigger a stress mediated DNA damage response that activates the p16 pathway. While the role of the p16/Rb pathway in

telomere biology and replicative senescence has been understood in human fibroblasts, it has not been understood in epithelial cells (19).

Since cancer is mostly a disease of epithelial cells, we believe our unique system of normal and spontaneously immortalized human breast epithelial cells should provide a good model system to examine the effects of tamoxifen and telomerase inhibitors. Inhibition and/or reversal of the immortal phenotype and other endpoints such as genomic instability, telomere stability, anchorage independent growth assays, should provide insights into the earliest stages of cancer development, leading to more effective cancer prevention measures.

Key Research Accomplishments

Original Statement Task 1. Inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors, Months 1-12:

- The immortalized HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Cells were lysed and assayed for telomerase activity and telomere length using established laboratory protocols.
- Method of growth inhibition was determined as apoptotic due to critically shortened telomeres.
- Verified that oligonucleotide phosphoramidates are efficient telomerase inhibitors and potential therapeutic agents.
- Microarray data revealed change in transcriptional profiling with telomerase inhibition.

Original Statement Task 2. Prevent the spontaneous immortalization of Li-Fraumeni Syndrome Human Mammary Epithelial Cells using telomerase inhibitors, originally Months 12-24:

- The HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Approximately ten population doublings before cells enter crisis, fluctuation analyses were performed.
- The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors.
- Microarray data revealed change in transcriptional profiling after tamoxifen treatment.

Original Statement Task 3. Understand the fate of breast epithelial cells with different p53 and pRB status when treated with telomerase inhibitors, Months 24-36:

- Breast epithelial cells of different p53 and pRB status were treated with telomerase inhibitors.
- Cell growth was monitored and cells were collected telomerase activity, telomere length, and protein analyses.
- Apoptosis and senescence assays were performed to determine fate of cells.
- Effects of telomerase inhibition are not dependent upon p53 and pRB status.

Reportable Outcomes

Employment received based on experience/training supported by this award:

Associate Member of the Cancer Center and Assistant Professor, Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana (Fall 2003)

Publications:

1. Herbert, B.-S., Pitts, A.E., Baker, S.I., Hamilton, S.E., Wright, W.E., Shay, J.W., and D.R. Corey. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. ***Proc Natl Acad Sci*** 96:14276-14281, 1999.

2. Herbert, B.-S., Wright, A.C., Passons, C.M., Kopelovich, L., Ali, I., Wright, W.E., and J.W. Shay. Effects of Chemopreventive and anti-telomerase agents on the spontaneous immortalization of breast epithelial cells. ***J Natl Cancer Inst*** 93:39-45, 2001.

3. Herbert, B.-S., Wright, W.E., and J.W. Shay. Telomerase and breast cancer. ***Breast Cancer Research*** 3:146-149, 2001.

4. Gryaznov, S., Pongracz, K., Matray, T., Schultz, R., Pruzan, R., Aimi, J., Chin, A., Harley, C., Shea-Herbert, B., Shay, J., Oshima, Y., Asai, A., and Y. Yamashita. Telomerase inhibitors- Oligonucleotide phosphoramidates as potential therapeutic agents. ***Nucleosides, Nucleotides & Nucleic Acids*** 20 (4-7):401-410, 2001.

5. Herbert, B.-S., Pongracz, K., Shay, J.W., and S.M. Gryaznov. Oligonucleotide N3'→P5' phosphoramidates as efficient telomerase inhibitors. ***Oncogene*** 21:638-642, 2002.

6. Herbert, B.-S., Wright, W.E., and J.W. Shay. $p16^{INK4a}$ inactivation is not required to immortalize human mammary epithelial cells. ***Oncogene*** 21:7897-7900, 2002.

7. Herbert, B.-S., Pearce V.P., Hynan, L.S., LaRue D.M., Wright, W.E., Kopelovich, L., and J.W. Shay. A peroxisome proliferator-activated receptor-gamma agonist and the p53-rescue drug CP-31398 inhibit the spontaneous immortalization of breast epithelial cells. ***Cancer Research*** 63:1914-1919, 2003.

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Abstracts:

1. Pitts, A.E., Baker, S.I., Herbert, B.-S., Shay, J.W., and D.R. Corey. 2'-O-Methyl RNA oligonucleotides directed agains the human telomerase template potently and selectively

inhibit telomerase activity, eroding telomeres and slowing growth. Cold Spring Harbor Meeting on Telomeres and Telomerase. March 25-28, p.124, 1999.

2. Herbert, B.-S., Kopelovich, L., Ali, I., Wright, W.E., and J.W. Shay. Telomerase inhibitors prevent the spontaneous immortalization of breast epithelial cells from individuals predisposed to breast cancer and induce apoptosis in immortal cells. DoD Breast Cancer Research Program Era of Hope Meeting. June 8-12, 2000.

3. Pongracz, K., Herbert, B.-S., Matray, T., Pruzan, R., Chin, A., Yamashita, Y., Harley, C., Shay, J.W., and S. Gryaznov. Inhibitors of telomerase- oligonucleotide N3'→P5' phosphoramidates. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

4. Herbert, B.-S., Steinert, S., Wright, W.E., and J.W. Shay. Telomerase inhibitors induce apoptosis in immortal human cells and prevent the spontaneous immortalization of breast epithelial cells from individuals predisposed to breast cancer. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

5. Corey, D.R., Herbert, B.S., Pitts, A.E., Baker, S.I., Wright, W.E., and J.W. Shay. Inhibtion of Human Telomerase in Immortal Human Cells Leads to Progressive Telomere Shortening and Cell Death. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

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Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death

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The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase and that telomerase inhibitors represent a class of chemotherapeutic agents. Herein, we examine the effects of inhibition of telomerase inside human cells. Peptide nucleic acid and 2′-*O*-MeRNA oligomers inhibit telomerase, leading to progressive telomere shortening and causing immortal human breast epithelial cells to undergo apoptosis with increasing frequency until no cells remain. Telomere shortening is reversible: if inhibitor addition is terminated, telomeres regain their initial lengths. Our results validate telomerase as a target for the discovery of anticancer drugs and supply general insights into the properties that successful agents will require regardless of chemical type. Chemically similar oligonucleotides are in clinical trials and have well characterized pharmacokinetics, making the inhibitors we describe practical lead compounds for testing for an antitelomerase chemotherapeutic strategy.

Human telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the ends of telomeres (1, 2). Telomerase activity has been found in almost all human tumors but not in adjacent normal cells (3, 4). This correlation has led to the hypotheses that reactivation of telomerase is necessary for sustained cell proliferation in many tumors and that telomerase is an exceptional target for a class of chemotherapeutic agents that act by an unidentified mechanism (5). Supporting these hypotheses is the observation that early-stage neuroblastomas have little or no telomerase activity, and this minimal activity generally correlates with a favorable outcome, whereas the late-stage disease has high telomerase activity and correlates with a poor outcome (6). A similar linkage between telomerase activity and poor clinical outcome has been reported for ordinary meningiomas (7), and other studies have suggested that telomerase activity is correlated with the pathologic stage (8–11) or tumor aggressiveness (11, 12).

Additional evidence for the importance of telomerase activity for sustained cell growth comes from studies of mice that lack the mouse telomerase RNA component (mTR). Depending on their genetic backgrounds, these mice survived for four to six generations with few detectable phenotypic changes (13, 14); however, by the seventh generation, highly proliferative organ systems like the testis, bone marrow, and spleen appeared abnormal, and the mice were no longer able to reproduce (15). In some genetic backgrounds, an increased incidence of neural tube defects limited viability after only four generations (14). Other studies of these mice revealed shortened life spans, an increased incidence of chromosomal abnormalities, and a slight increase in spontaneous malignancies (16). The long lag between loss of telomerase activity and a detrimental phenotype suggests that telomerase will be a difficult target for effective chemotherapy, but the mice used in these experiments possessed much longer telomeres than those found in cancerous human cells, and inhibition of telomerase in human cancer would be expected to produce effects more rapidly. Furthermore, recent reports of the effect of the mTR deletion in different genetic backgrounds (17, 18) suggest that the role of telomerase in cancer development could be highly context dependent (19).

Mutation of the RNA component of telomerase of *Tetrahymena* (20), *Kluyveromyces lactis* (21), *Saccharomyces cerevisiae* (22), and human cells (23) also leads to decreased cell proliferation, whereas expression of antisense RNA complementary to the human telomerase RNA component (hTR) caused decreased proliferation of HeLa cells after 23 to 26 doublings (24). Conversely, transfection of cells with the gene encoding the human telomerase reverse transcriptase component (hTERT) and subsequent expression of active telomerase have been shown to extend the life spans of normal human fibroblasts and epithelial cells (25–27). Most recently, expression of telomerase in conjunction with expression of simian virus 40 large T oncoprotein and an oncogenic allele of H-ras has been shown to promote tumorigenic conversion of normal human cells (28). Thus, the lack of telomerase expression seems to curb growth of rapidly proliferating cells eventually, whereas an increase in telomerase permits indefinite proliferation.

A potential concern for the telomerase–cancer connection is the observation that a few rare tumors (29) and some experimentally immortalized cell lines (30) lack detectable telomerase activity. Thus, although telomerase activity can confer extended life span to cells, other mechanisms may also exist. A pathway termed ALT (alternative lengthening of telomeres) has been proposed to account for this phenomenon. Evidence for the existence of this pathway is found in yeast in which the gene for est1, a protein involved in telomere-length maintenance, has been deleted (31). The cells divide normally until telomeres shorten sufficiently to affect proliferation. At this stage, most cells die, but some rare survivors continue to proliferate through a recombination mechanism to maintain telomere length.

Telomerase will be an unusually challenging target for drug development because of the long lag period expected before telomeres would shorten sufficiently to produce detrimental effects on cell growth and because of the possibility of alternate mechanisms for maintenance of telomeres. Because of this uncertainty, discovery of the potential of telomerase as a target for human therapy requires development of potent and selective synthetic inhibitors and their testing inside cells. To confirm action through a telomerase-dependent mechanism, inhibitors

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Abbreviations: mTR and hTR, mouse and human telomerase RNA component; hTERT, human telomerase reverse transcriptase component; PNA, peptide nucleic acid; HME, human mammary epithelial; TRF, terminal restriction fragment.

*B.-S.H. and A.E.P. contributed equally to this work.

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must meet the following criteria: (*i*) inhibitors should reduce telomerase activity but, initially, should not affect cell growth rates; (*ii*) addition of inhibitors should lead to progressive shortening of telomeres with each cell division; (*iii*) addition of inhibitors should cause cells to die or undergo growth arrest; (*iv*) the time necessary to observe decreased proliferation should vary depending on initial telomere length; and (*v*) chemically related molecules that do not inhibit telomerase activity should not cause decreased cell proliferation or telomere shortening.

Herein, we examine how inhibition of telomerase by exogenously added molecules affects cellular phenotypes. We observe that 2′-*O*-MeRNA and peptide nucleic acid (PNA) oligomers complementary to the template region of hTR inhibit telomerase and cause telomeres to shorten and that extended treatment with 2′-*O*-MeRNA promotes cell death through apoptosis. Inhibition of cell proliferation suggests that oligonucleotides are a viable approach to antitelomerase therapy, and our results should encourage and guide further testing of other approaches to telomerase inhibition such as nucleotide analogues (32), G quadruplex interactive agents (33, 34), or other small molecules.

Materials and Methods

Cell Lines. The human mammary epithelial (HME) cells used for these experiments were spontaneously immortalized from an epithelial culture derived from normal breast tissue from a patient with Li-Fraumeni syndrome (35). Cells were grown in serum-free medium (MCDB from GIBCO) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 10 ng/ml epidermal growth factor (GIBCO), 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin, and 50 μg/ml gentamicin (Sigma). The medium was changed every 2–3 days. Cells were used between population doublings 100 and 150. The prostate-tumor-derived DU145 cells were maintained in DMEM containing 10% (vol/vol) FCS, 500 units/ml penicillin (Sigma), and 0.1 μg/ml streptomycin (Sigma) and incubated at 5% CO_2 at 37°C. Detection and quantitation of apoptosis by flow cytometry were performed with the ApoAlert Annexin V Apoptosis kit (CLONTECH).

Oligonucleotides and PNAs. We purchased 2′-*O*-MeRNA oligonucleotides from Oligos Etc. and Oligo Therapeutics (Wilsonville, OR). The sequence of the match phosphorothioate modified 2′-*O*-MeRNA is 5′-CAGUUAGGGUUAG-3′; the mismatch sequence is 5′-CAGUUAGAAUUAG-3′, where the underlined nucleotides possess phosphorothioate linkages. Match and mismatch PNAs (36) of similar sequences and nontemplate directed PNA AGCGGGCCAGCAGCTG were synthesized automatically with a PerSeptive Biosystems (Framingham, MA) Expedite 8909 Synthesizer by using Fmoc protocols and reagents obtained from PE Biosystems. PNAs were purified by HPLC and characterized by matrix-assisted laser desorption time-of-flight mass spectrometry by using a Voyager-DE mass spectrometry workstation (PE Biosystems) as described (37). The sequence of the match PNA is Gly-CAGTTAGGGTTAG-Lys; the sequence of the mismatch is Gly-CAGTTAGAATTAG-Lys. DNA oligonucleotides for transfection of PNA/DNA complexes were obtained from Life Technologies (Gaithersburg, MD). The sequence of the DNA oligonucleotide complexed to the match PNA is (5′–3′) TCTAACCCTAA; the sequence of the DNA oligonucleotide complexed to the mismatch PNA is (5′–3′) TCTAATTCTAA.

Uptake of 2′-*O*-MeRNA and PNA into Cells. HME50-5E and HME50-hTERT cells were transfected with 2′-*O*-MeRNA and mismatch control oligomers after the FuGENE6 Transfection Reagent protocol (Roche Molecular Biochemicals). Briefly, 5×10^4 cells were allowed to adhere overnight in appropriate media. The next day, the oligomers were incubated for 15 min with the previously combined FuGENE6 Transfection Reagent and PBS. After changing to fresh medium, the oligomer mixture was added dropwise to the cells. Cells were harvested after 4 days, counted, and replated, and telomerase activity was determined.

DU145 cells were plated at 25,000 cells per well in a 24-well plate in DMEM supplemented with 10% (vol/vol) FCS, 500 units/ml penicillin, and 0.1 μg/ml streptomycin. For DNA/PNA transfections 100 μM PNA was hybridized with 109 μM appropriate DNA oligonucleotide in 0.5× PBS. After allowing the cells to adhere, they were transfected with 2.0 μl (7 μg/ml) of Lipofectamine (Life Technologies) and 0.5 μM 2′-*O*-MeRNA oligonucleotide (38) or 1 μM PNA/DNA complex (39) in 200 μl of total Opti-Mem (Life Technologies) according to the manufacturer's directions. After 6 h at 37°C, the transfecting mixture was removed, and medium without antibiotics and with 20% (vol/vol) serum was added. Cells were then harvested 12–15 h later after three washes with PBS and treatment with trypsin or allowed to grow for 3 days. Cells were harvested after 3 days, counted, replated, and assayed for telomerase activity.

Measurement of Telomerase Activity and Telomere Length. Telomerase activity was measured by telomere repeat amplification protocol by using the TRAPeze telomerase detection kit (Intergen, Purchase NY; ref. 40). After the extension of the substrate by telomerase, the products were amplified by PCR in the presence of an ^{32}P-end-labeled TS primer, resolved on 10% polyacrylamide gels, and revealed by exposure to a PhosphorImager cassette (Molecular Dynamics). Telomerase activity was calculated as the ratio of the intensity of telomerase ladders to the intensity of the 36-bp internal standard. Percentage of inhibition was calculated by comparing telomerase activity of oligomer-treated cells with telomerase activity of cells treated with lipid alone. The levels of telomerase activity were within the linear range of the TRAP assay (40). Mean telomere length was evaluated by using telomere restriction fragment analysis, a variation of standard Southern analysis, and was quantitated as described (41). Digested samples were resolved on a 0.7% agarose gel and hybridized to a telomeric probe [[^{32}P](T-TAGGG)$_4$ oligonucleotide].

Results

Effect of 2′-*O*-MeRNA on Cell Proliferation. We used cationic lipids to introduce 2′-*O*-MeRNA complementary to the template region of hTR into the immortalized human cell lines HME50-5E and DU145. We also introduced 2′-*O*-MeRNA into HME50-hTERT cells, a cell line generated by infecting preimmortal HME50 cells with the gene encoding hTERT. A 2′-*O*-MeRNA containing mismatched bases was employed as a control for the sequence-specificity of inhibitor action. Analysis of telomerase inhibition by TRAP indicated that the complementary 2′-*O*-MeRNA oligomer blocked more than 95% of telomerase activity 1 day after transfection, and more than 70% of activity was inhibited 3 days after transfection (Fig. 1). The 2′-*O*-MeRNA oligomers used in these studies are stable to degradation inside cells (38); thus, it is likely that the reduced inhibition is due to dilution of inhibitor as the cell population increases.

To determine whether inhibition of telomerase would ultimately limit proliferation, we transfected HME50-5E, DU145, and HME50-hTERT cells with 2′-*O*-MeRNA oligomers at 3- to 4-day intervals for 120 days. Because of the extreme length of these experiments, stringent precautions were taken to avoid contamination of cultured cells, and all experiments were completed successfully without interruption. After 15–25 days, proliferation of HME50-5E cells treated with the complementary oligomer began to slow, and after 110 days, no treated cells remained (Fig. 2*a*). The growth of cells treated with the control

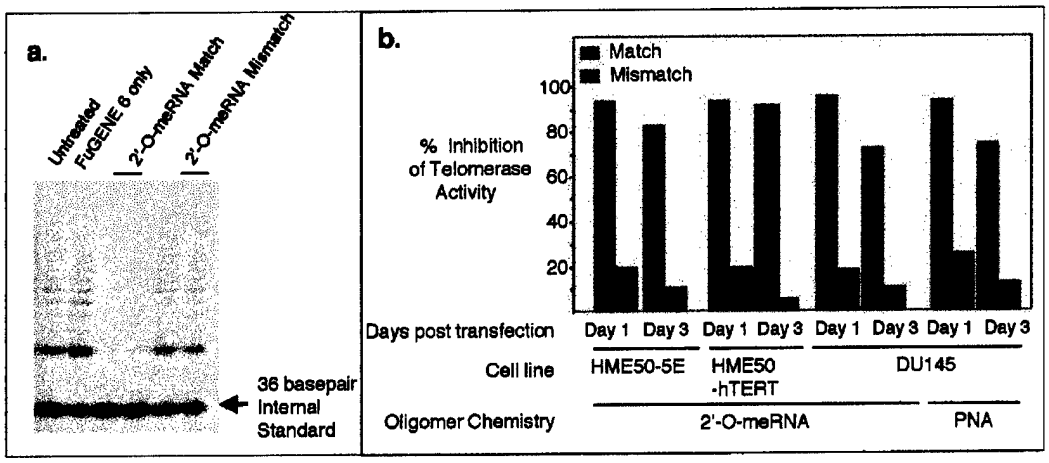

Fig. 1. (*a*) Inhibition of telomerase activity by 2′-*O*-MeRNA oligomers delivered into HME50-5E cells by using FuGENE6 lipid, measured 3 days after transfection. Similar results were observed on introduction of 2′-*O*-MeRNA oligomers into HME50-hTERT cells and on introduction of 2′-*O*-MeRNA or PNA oligomers into DU145 cells. (*b*) Inhibition of telomerase activity in HME50-5E and DU145 cells by 2′-*O*-MeRNA and PNA oligomers as detected by the TRAP assay. HME50-5E and DU145 cells were collected 1 and 3 days after transfection with 2′-*O*-MeRNA or PNA oligomers. Telomerase activity was quantitated as described (38).

oligomer containing mismatch bases was not affected. During the first 60 days after initial transfection, less than 5% of cells treated with the fully complementary 2′-*O*-MeRNA underwent apoptosis. By 80 days, however, 14% of cells treated with the match oligomer were observed to undergo apoptosis, and this percentage increased to 56% after 100 days with cell viability lost after 110 days (Fig. 3). By contrast, cells treated with lipid alone or the 2′-*O*-MeRNA oligomer containing mismatched bases underwent apoptosis at a rate of 2–3% throughout the entire experiment. To confirm that the effects of inhibitor addition were reproducible, treatment of HME50-5E cells with 2′-*O*-MeRNA was repeated, and a similar decrease in proliferation was observed.

Addition of complementary 2′-*O*-MeRNA to DU145 cells caused proliferation of cells to begin to slow after 60 days (Fig. 2*b*). By the end of the experiment (after 120 days), these cells had undergone 20 fewer population doublings than had cells treated with mismatch-containing oligomer. HME50-5E cells have a mean terminal restriction fragment (TRF) length of 2,000 bp, whereas DU145 cells possess a mean length of 3,600 bp. TRF length corresponds qualitatively to telomere length, and the finding that proliferation of treated HME50-5E cells decreases more dramatically than does cell proliferation of treated DU145 cells may be due to HME50-5E cells possessing shorter telomeres. Consistent with this hypothesis, HME50-hTERT cells have a much longer mean TRF length (7.6 kilobases) than either HME50-5E or DU145 cells, and we observed no significant reduction in their growth rates during the 120-day treatment period (Fig. 2*c*).

Telomere Shortening On Addition of 2′-*O*-MeRNA. To support the hypothesis that the decrease in cell proliferation is caused by the 2′-*O*-MeRNA oligomer through a telomerase-dependent mechanism rather than being caused by long-term toxicity that is

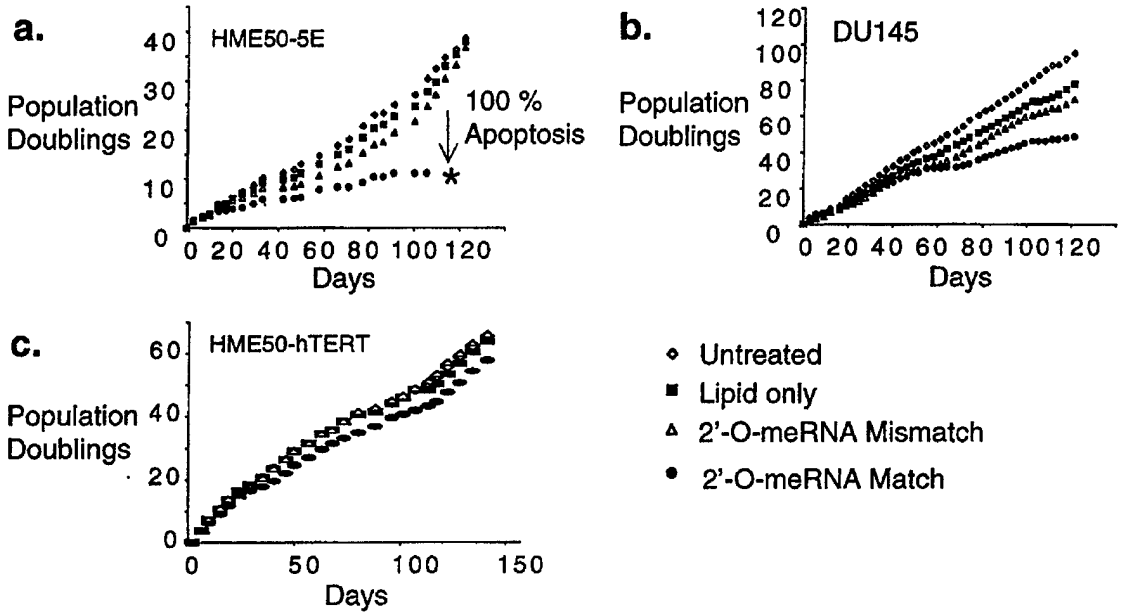

Fig. 2. Effects on cell growth after long-term transfection with match or mismatch 2′-*O*-MeRNA. (*a*) HME50-5E cells transfected with 2′-*O*-MeRNA oligomers. (*b*) DU145 cells transfected with 2′-*O*-MeRNA oligomers. (*c*) HME50-hTERT cells transfected with 2′-*O*-MeRNA oligomers.

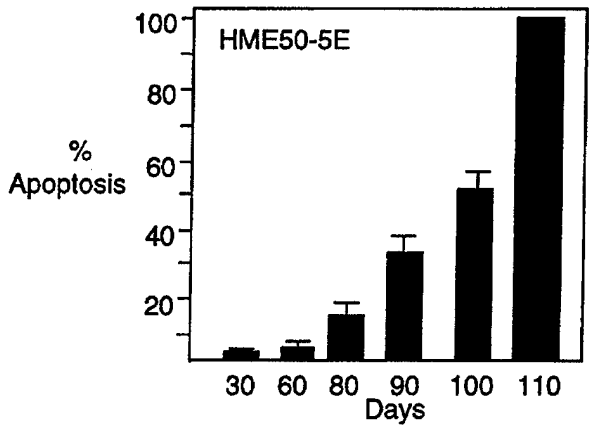

Fig. 3. Increase of apoptosis of HME50-5E cells after repeated transfection with fully complementary 2′-*O*-MeRNA. Levels of apoptosis were measured by staining with 4′6-diamidino-2-phenylindole followed by microscopy. Background apoptosis of cells that were untreated or that were treated with 2′-*O*-MeRNA containing mismatched bases was 2–3%. Levels of apoptosis were confirmed by flow cytometry with the ApoAlert Annexin V Apoptosis kit (CLONTECH).

independent of telomerase inhibition, we monitored the effect of inhibition on telomere length. Within 60 days of treatment, the mean telomere length of HME50-5E cells treated with 2′-*O*-MeRNA complementary to hTR was reduced from 2,000 to 1,700 bp (Fig. 4*a*). This decrease in measured TRF length may be an underestimate of the total loss of chromosomal DNA, because the TRF assay does not monitor erosion of subtelomeric regions and because relatively little telomeric DNA remained to hybridize with radiolabeled probe. The TRF length of cells treated with 2-*O*-MeRNA containing mismatches relative to hTR remained at 2,000 bp, and the signal intensity of probe hybridization was undiminished.

We also examined the mean TRF length of DU145 cells that had been treated with 2′-*O*-MeRNA oligomers for 76 days. Because DU145 cells possess longer telomeres, we were able to observe a dramatic erosion of telomere length, with the mean TRF length of cells treated with the 2′-*O*-MeRNA complementary to the template region of hTR decreasing from 3,600 bp to 2,200 bp (Fig. 4*b*). As observed with HME50-5E cells, the signal intensity of probe hybridization was greatly reduced because of the reduction in telomeric repeats. We also examined the effect of inhibitor treatment on HME50-hTERT cells and found that TRF length decreased from 7,600 to 6,800 bp after 75 days (results not shown).

The 2′-*O*-MeRNA inhibitor contained four phosphorothioate linkages, a chemical moiety noted for its propensity to produce misleading cellular effects that seem sequence-specific but are actually unrelated to binding to the intended target (42). Demonstrating that decreased cell proliferation derives from a telomerase-dependent mechanism involving binding of inhibitor to the RNA template is a critical prerequisite for embarking on lengthy studies in animals and humans. Therefore, to provide further evidence that the reduction of telomere length was due to Watson–Crick recognition of the template region of hTR, we tested the effects of addition of match and mismatch PNAs (36).

PNAs are ideal agents for confirming the specificity of oligonucleotides, because they possess a neutral amide backbone and have a much lower propensity for making unwanted interactions with macromolecules that bind the repetitive anionic linkages of DNA and RNA. PNAs cannot be introduced into cells by direct complexation with cationic lipid, because PNA backbone linkages are uncharged; however, PNAs can be hybridized to DNA oligonucleotides and introduced into cells as cargo on complexation of DNA with lipid (39). Addition of a PNA that was complementary to the hTR template to DU145 cells inhibited over 90% of telomerase activity after 1 day (Fig. 1*b*) and caused telomere shortening similar to that caused by addition of complementary 2′-*O*-MeRNA (Fig. 4*b*). A PNA directed to a nontemplate region of hTR inhibited only 50% of telomerase activity after 1 day and did not cause telomeres to shorten (results not shown).

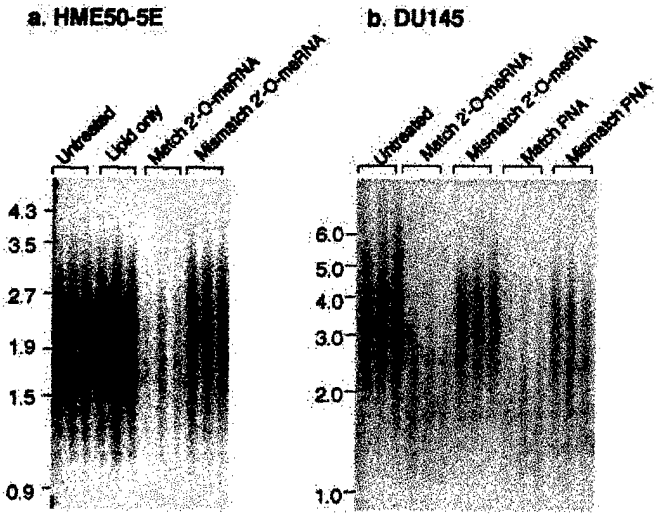

Fig. 4. Measurement of telomere restriction fragment length (TRF) in HME50-5E and DU145 cells treated with 2′-*O*-MeRNA and PNA oligomers. (*a*) HME50-5E cells that had been treated with 2′-*O*-MeRNA oligomers for 60 days, with the results of independent experiments shown in triplicate. (*b*) DU145 cells treated with 2′-*O*-MeRNA and PNA oligomers for 76 days, with the results of independent experiments shown in triplicate. In parts *a* and *b*, the signal intensity in the lanes showing the outcome of treatment with fully complementary oligomer is weak, because telomeres have eroded and few telomeric repeats remain to hybridize with radiolabeled probe. Equivalent amounts of chromosomal DNA were loaded in each lane. TRF lengths are expressed as kilobase pairs.

Effects of Terminating Inhibitor Addition. An inherent advantage of using synthetic inhibitors to reduce gene expression rather than genetic knockouts is that the phenotypic effects of regaining function can be evaluated by terminating inhibitor addition. After 76 days, we stopped adding inhibitor to DU145 cells that had been treated with fully complementary or mismatch-containing 2′-*O*-MeRNA or PNA oligomers. After 3 weeks, we noted that the eroded telomeres of cells treated with fully complementary oligomers had returned to approximately their initial lengths (Fig. 5). Telomeres remained near their initial lengths when measured a second time at 5 weeks. We also evaluated the effect of inhibitor withdrawal on proliferation of HME50-5E cells that had been treated with the fully complementary 2′-*O*-MeRNA until population growth was static. We observed that, within 3 weeks of terminating inhibitor treatment, previously static HME50-5E cells regained the ability to grow at the same rate as cells treated with mismatch-containing 2′-*O*-MeRNA.

Discussion

Telomerase as a Target for Chemotherapy. Cancer remains a major cause of morbidity and mortality in spite of substantial progress toward understanding the molecular basis of the disease. The discovery of new drugs is urgent, and telomerase inhibitors have the potential to provide an additional option for chemotherapy. Telomerase inhibitors might not only limit growth of human

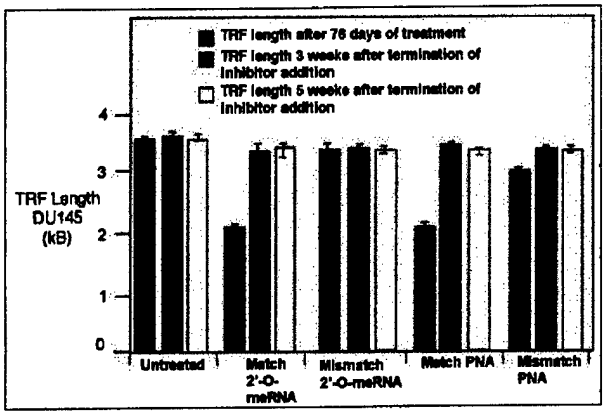

Fig. 5. The TRF length of DU145 as a function of inhibitor addition. TRF lengths were measured after 76 days of addition of fully complementary or mismatch-containing 2′-*O*-MeRNA or PNA oligomers. TRF lengths were then measured again 3 or 5 weeks after terminating oligomer addition. kB, kilobase.

tumors directly but might also act in a synergistic fashion with existing inhibitors and amplify their efficacy. For example, after initial chemotherapy or surgery, telomerase inhibitors might be used in an adjuvant setting to limit the recovery of residual cancer cells, making them more susceptible to attack by the immune system or killing by existing chemotherapeutic agents. The use of telomerase inhibitors is particularly attractive for situations of ongoing cell turnover, as occurs with tumor-static antiangiogenesis inhibitors. Systemic administration of telomerase inhibitors will also inhibit telomerase in normal stem cells. This inhibition may result in few side effects, because the relatively short telomeres of tumors may erode to a critical length before irreversible harm is done to other cells.

Oligonucleotides as Telomerase Inhibitors. Telomerase is an ideal target for oligonucleotides, because its RNA template is essential for activity and is intrinsically accessible to binding by nucleic acids. Oligonucleotides are being tested in 13 ongoing clinical trials (43), and recently, one oligonucleotide, Fomivirsen, has been approved for the treatment of cytomegalovirus retinitis (44). In addition, 2′-*O*-MeRNA and other 2′-*O*-alkyl-derivatized oligomers bind more tightly to complementary RNA sequences than do analogous DNA oligomers and have improved resistance to degradation by nucleases, reducing the need for phosphorothioate linkages and improving the selectivity of antisense effects (45, 46); 2′-*O*-alkyl RNA is currently being used in two phase II clinical trials including one trial directed against the R1-α subunit of protein kinase A, an application similar to the use of telomerase inhibitors to prevent tumor regrowth. Experience with 2′-*O*-alkyl oligomers in clinical trials, combined with our observation of both decreased cell proliferation and increased apoptosis, will encourage the testing of antitelomerase oligonucleotides in animals and humans.

Phenotypic Effects Are Mediated by a Sequence-Specific, Telomerase-Dependent Mechanism. Use of oligonucleotides to affect cellular phenotypes is often met with skepticism, because non-sequence-specific effects and misleading phenotypes have plagued their use. Tu *et al.* (47) have performed a comprehensive survey of the literature, leading Stein (48) to estimate that non-sequence-selective effects may be at least partially implicated in results reported in 94% of the 2,026 studies published through 1997. Indeed, we have previously observed that oligonucleotides that contain uniform phosphorothioate oligonucleotides inhibit telomerase in a non-sequence-specific fashion, presumably through binding to hTERT (38).

We offer multiple independent lines of evidence in the present study that the effects we observe are due to Watson–Crick recognition of telomerase. Treatment with match 2′-*O*-MeRNA and PNA oligomers cause much greater decreases in telomere length and cell proliferation than does treatment with oligomers that contain mismatched bases or treatment with a less efficient PNA inhibitor directed to a nontemplate region of hTR. The fact that inhibition of telomerase has a more rapid and pronounced effect on proliferation of HME50-5E cells, which have shorter telomeres than DU145 cells, supports a mechanism of decreased proliferation involving telomere shortening rather than nonselective toxicity. Similarly, even though the genetic background of HME50-hTERT cells is similar to that of HME50-5E cells, proliferation of treated HME50-hTERT cells did not decrease, presumably because their initial telomere length of HME50-hTERT cells is approximately 4,000 bp longer. PNA and 2′-*O*-MeRNA oligomers cause similar reductions in telomere length, suggesting that the effects are caused by Watson–Crick recognition rather than by non-base-pair-mediated contacts with backbone phosphodiester or phosphorothioate linkages. The use of PNAs to confirm the mechanism of oligonucleotide action has important general implications for the use of oligonucleotides within cells, because validating the mechanism of oligonucleotide action is a key problem for antisense research. If neutral and anionic oligomers produce the same effects, they likely derive from specific Watson–Crick base pairing rather than misleading non-sequence-specific interactions. Recent work with PNAs introduced into cells by electroporation has shown decreased cell viability, supporting our findings (49).

General Implications for Development of Telomerase Inhibitors. Typically, when a putative antiproliferative agent is applied to cells, an effect is observed within hours or days. Telomerase is an unusually challenging target for drug discovery, because a cellular response that depends on telomere shortening will require weeks to become apparent. Although this outcome should not have serious consequences for administration of proven drugs to patients accustomed to chronic treatment of residual disease, it greatly complicates the assays needed to develop and test such drugs. Our primary finding is that telomerase is a viable target for synthetic agents aimed at controlling proliferation of immortal human cells. No evidence for adoption of an ALT (alternative lengthening of telomeres) pathway for telomere maintenance was observed, suggesting that this pathway may not be readily adopted by some immortal cell types. The decreased proliferation we observe should encourage further development and testing of other promising classes of compounds such as nucleoside derivatives directed to hTERT (32) or small molecules designed to disrupt telomere synthesis by promoting G quadruplex formation (33, 34).

Our results have broad implications for how telomerase inhibitors must act within cells to be effective and on how protocols for animal and clinical testing must be designed. We observe telomere shortening and decreased cell proliferation, even though between 5% and 30% of telomerase activity remained after treatment with inhibitor. However, addition of a PNA directed to a nontemplate region of hTR, which is a less potent inhibitor, does not lead to telomere erosion, and telomeres rapidly regain their lengths once inhibitor addition is terminated. Taken together, these results suggest that, although obtaining a striking phenotypic effect does not require full inhibition of telomerase, potency remains an important design consideration. Inhibitors will be most effective against tumor cells with short telomeres, and inhibition of telomerase must be maintained at a sustained high level over time to prevent the rapid regrowth of telomeres. Regrowth to initial telomere length

on cessation of inhibitor addition suggests that there is a "set point" for telomere length in human cells and that the telomeres of stem cells that erode during therapy may recover once treatment is terminated. HME50-5E cells were derived from HME50 preimmortal cells that had one mutant p53 allele and lost the second allele during spontaneous immortalization, whereas DU145 cells have two mutant alleles, indicating that functional p53 is not necessary for the reduced proliferation of these cell lines on treatment with telomerase inhibitors.

Conclusions. The potential for telomerase to be a target for anticancer chemotherapy has engendered a debate that evokes both great enthusiasm and great skepticism. Our findings indicate that telomerase is a viable target for chemotherapeutic drugs and are important for two reasons. The first is that our data will encourage investment in the demanding long-term studies needed to discover and test other classes of inhibitors and guide the design of protocols that optimize the likelihood of obtaining definitive results in animals. The second is that oligonucleotides seem to be excellent candidates for antitelomerase drugs in their own right. The exposed RNA template of hTR makes telomerase an ideal target for oligonucleotides, and the presence of similar oligonucleotides in clinical trials suggests that it should be possible to test potent antitelomerase oligonucleotides *in vivo* in the near future.

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REPORTS

Effects of Chemopreventive and Antitelomerase Agents on the Spontaneous Immortalization of Breast Epithelial Cells

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Background: **Activation of telomerase is an early event in the development of breast and other cancers that may lead to cell immortalization, a critical and rate-limiting step in cancer progression. Breast epithelial cells from women with Li–Fraumeni syndrome (LFS) immortalize spontaneously and reproducibly in culture. We, therefore, tested whether immortalization of these cells could be prevented by treating them with chemopreventive agents and by inhibiting telomerase activity.** ***Methods:*** **Noncancerous, preimmortal breast epithelial cells derived from a patient with LFS were treated for 3 months with nontoxic concentrations of the chemopreventive agents oltipraz, difluoromethylornithine, tamoxifen, and retinoic acid or with two different telomerase inhibitors. The frequency of spontaneous immortalization of LFS-derived cells was estimated by an approach based on fluctuation analyses. Statistical analyses were two-sided.** ***Results:*** **The frequency of spontaneous immortalization events of LFS-derived breast epithelial cells was reduced by long-term treatment with retinoic acid ($P<.001$) or tamoxifen ($P<.05$) compared with solvent-treated cells. The frequency of immortalization was also reduced by treating LFS-derived cells with an antitelomerase antisense oligonucleotide ($P<.001$) or by inducing the cells to express a dominant negative mutant of telomerase ($P<.025$) compared with cells treated with a control oligonucleotide or with empty vector, respectively.** ***Conclusions:*** **Treatment of preimmortal LFS breast epithelial cells with chemopreventive and antitelomerase agents decreased the frequency of spontaneous immortalization *in vitro*. These studies validate the application of a new cell culture model system to screen the effects of novel chemopreventive agents by use of cell immortalization as an end point. The results also suggest that the telomerase ribonucleoprotein complex may be an important molecular target for breast cancer prevention. [J Natl Cancer Inst 2001;93: 39–45]**

Li–Fraumeni syndrome (LFS) is an autosomal dominant trait that results in childhood sarcomas and early-onset breast cancer. LFS is frequently characterized by inherited mutations in the tumor suppressor gene, p53 (also known as TP53) *(1)*. Alterations in the p53 gene are thought to lead to genomic instability by allowing uncontrolled cellular proliferation, which perpetuates further instability *(2)*. Breast tumors are the most prevalent cancer among women in LFS families; 25% of the breast cancers are diagnosed in women under age 30 years and 89% in women under age 50 years *(1,3,4)*. The molecular mechanisms that lead to the specific increase in the incidence of breast cancer as opposed to other cancers in LFS families are not understood *(5,6)*.

The cell immortalization that leads to breast cancer in LFS patients presumably involves the activation of telomerase. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to the ends of chromosomes *(7)*. In most normal human cells, with the exception of germ cells and stem cells, the chromosomes progressively shorten their telomeres with each cell division because telomerase is not active. Cellular senescence occurs when cells with critically shortened chromosomes undergo a permanent growth arrest.

Cell immortalization is thought to occur when the progressive telomere shortening that normally occurs in most cells is prevented by the reactivation or increased expression of telomerase, although other mechanisms that stabilize and maintain telomeres are also possible *(8)*. Indeed, telomerase activity was detected in nearly all (90%) cancers tested *(9,10)*. Immortal cells emerge by escaping crisis, which is a period of balanced cell growth and cell death followed by a decrease in the total number of surviving cells. If telomerase activation is necessary for cell immortalization, then telomerase may provide a target for cancer treatment and prevention. Previous reports *(11–13)* have demonstrated that treatment of immortal and cancerous cell lines with antitelomerase agents can inhibit their growth, leading to apoptotic cell death in a p53-independent manner.

To study the effects of chemopreventive and antitelomerase agents on immortalization, it is necessary to examine cells that become immortal *in vitro*. However, the spontaneous immortalization of human cells in culture is an extremely rare event: It requires mutations in several genes, such as p16, p53, and pRb, and their cellular pathways that are involved in cellular senescence *(8,14,15)*. Although normal human breast epithelial cells *in vitro* never immortalize spontaneously, breast epithelial cells derived from LFS patients do so at a detectable and reproducible frequency of 5×10^{-7}. These cells, which initially are heterozygous for wild-type p53 and lack telomerase activity, spontaneously inactivate p16 and the remaining wild-type p53 allele *in vitro*, resulting in immortalized cultures that express telomerase *(16)*.

To investigate the nature of the immortalization process, we surveyed a panel of clinically validated chemopreventive agents for their effects on the spontaneous immortalization of LFS-derived breast epithelial cells *in vitro*. Because immortalization usually involves the activation of telomerase, we also used this model system to examine the effects of antitelomerase agents on breast epithelial cell immortalization. Telomerase activation occurs early in breast cancer progression; hence, it is an attractive target for the treatment and prevention of breast cancer *(10,17–20)*. We examined two antitelom-

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See "Notes" following "References."

erase agents: One is an antisense oligonucleotide complementary to the template region of human telomerase RNA, and the other is a dominant-negative mutant of the catalytic subunit of human telomerase. These studies provide a new model to screen novel chemopreventive agents that target cellular immortalization, a critical step in cancer progression.

Materials and Methods

Cell Culture

The human mammary epithelial (HME) cells used for these experiments were derived from the noncancerous breast tissue of a 31-year-old female diagnosed with LFS described previously *(16)*. These cells contain a germline mutation (Met133Thr) in the p53 gene that affects the conformation of the wild-type p53 protein *(16)*. Immunohistochemical analysis confirmed that these cells do not express the estrogen receptor-α (data not shown). The cells were grown in MCDB 170 media (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 10 ng/mL epidermal growth factor (Life Technologies, Inc.), and 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 5 μg/mL transferrin, and 50 μg/mL gentamicin (all from Sigma Chemical Co.; St. Louis, MO). The medium was changed every 2–3 days.

Treatment With Telomerase Inhibitors

Antitelomerase agents. Phosphorothioate-modified 2′-*O*-methyl RNA oligomers were purchased from Oligos Etc. (Wilsonville, OR). The sequence of the antisense RNA complementary to the template region of human telomerase RNA is 5′-CAGUUAGGGUUAG-3′; the sequence of the mismatched RNA is 5′-CAGUUAG*AA*UUAG-3′, where the underlined nucleotides possess phosphorothioate linkages and mismatched nucleotides are indicated by italics. The antisense and mismatched oligomers were introduced into cells every 4 days during the course of the study by transfection with FuGENE6-transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN).

Construction and expression of dominant-negative telomerase mutant. The D869A-hTERT complementary DNA (cDNA) is a derivative of the modified hTERT cDNA that contains a point mutation within the highly conserved reverse transcriptase motif that changes the aspartic acid residue at amino acid 869 to an alanine *(21)*. The D869A-hTERT cDNA was inserted into the *Eco*RI site of the retroviral vector pBABEpuro to create pBABEpuro D869A-hTERT *(22)*. The pBABEpuro D869A-hTERT and pBABEpuro (empty vector) were introduced separately into the mouse ecotropic packaging cell line PE501 *(23)* by electroporation. The ecotropic viruses that were produced were supplemented with polybrene (Sigma Chemical Co.) to a final concentration of 4 μg/mL and used to infect the mouse amphotropic packaging cell line PA317 *(23)*. Infected cells were cultured in the presence of 4 μg/mL puromycin (Sigma Chemical Co.) for 1 week to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector). The culture supernatants containing amphotropic viruses were then collected, supplemented with polybrene to a final concentration of 4 μg/mL, and used to infect HME cells. Infected cells were cultured in the presence of 150 ng/mL puromycin to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector).

Treatment With Chemopreventive Agents

HME cells were treated every 3 days for approximately 25 population doublings with the following clinically validated chemopreventive agents: oltipraz, difluoromethylornithine (DFMO), tamoxifen citrate, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid. Oltipraz, DFMO, tamoxifen citrate, and 9-*cis*-retinoic acid were obtained from the DCP Repository (McKesson BioServices, Rockville, MD). 13-*cis*-Retinoic acid was purchased from Sigma Chemical Co. Oltipraz, tamoxifen citrate, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid were dissolved in dimethyl sulfoxide and stored at −80 °C as 1000× stocks. DFMO was dissolved in HME culture media and stored at −80 °C as 1000× stocks. Chemopreventive agents were diluted to the appropriate working concentrations in HME culture media.

Fluctuation Analysis to Determine the Frequency of Immortalization

The frequency of immortalization was estimated by use of a fluctuation analysis as described previously *(16,24,25)*. Each clone of cells that emerged from crisis was counted as an independent immortalization event. Frequency is defined as the probability of obtaining an immortal cell line on the basis of the total number of cells plated at each passage, not on the number of cell divisions, and is calculated by dividing the total number of independent immortalization events among all dishes by the total number of cells plated. In this study, untreated, mock-treated, and treated cells were expanded to 10 culture dishes approximately 10 population doublings before they would have normally reached crisis *(24,25)* and were maintained at a constant density of 10^6 cells per dish. Once the cells reached crisis, they were harvested, replated, and re-treated at least once every 3 weeks until virtually no surviving cells remained or until the culture had a small focus of growing cells. Cells were considered to be immortal if they expressed telomerase and had undergone vigorous, postcrisis growth *(16)*. Each small focus of growing cells was counted as an immortal event; the total number of immortal events from each of the 10 dishes for each treatment was recorded. Frequency of immortalization was calculated by dividing the number of immortal events by the total number of cells plated (10×10^6 cells). For each set of treatments, we performed and averaged three fluctuation analyses at three separate times by use of the same starting populations of cells recovered from freezer stocks.

Measurement of Telomerase Activity

Cells were resuspended in lysis buffer (i.e., 10 m*M* Tris–HCl [pH 8.0], 1 m*M* $MgCl_2$, 1 m*M* EGTA, 1% Nonidet P-40, 0.25 m*M* sodium deoxycholate, 10% glycerol, 150 m*M* NaCl, and 5 m*M* β-mercaptoethanol) at a concentration of 1000 cells/μL, were incubated on ice for 30 minutes, and were then centrifuged at 14 000*g* for 20 minutes at 4 °C *(21,26)*. The resulting supernatants were used directly to detect telomerase activity (by use of 500 cell equivalents per assay) or were flash-frozen and stored at −80 °C. Telomerase activity was measured by use of the TRAP-eze Telomerase Detection kit (Intergen, Purchase, NY), with the telomerase substrate (TS) primer (5′-AATCCGTCGAGCAGAGTT-3′) as the substrate. After the extension of the substrate by telomerase (for 30 minutes at room temperature), the extension products were amplified by polymerase chain reaction by use of the TS primer end labeled with ^{32}P, resolved on a 10% polyacrylamide gel, and revealed by exposure to a PhosphorImaging cassette (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Each dish was counted as having an immortalization event occur or not having an immortalization event occur. Because the data were collected as three different experiments for the cells treated with chemopreventive agents (Table 1) and with antitelomerase agents (Table 2) and the proportion of dishes with an immortal event were small in some treatments, a two-tailed Fisher's exact test, using the number of dishes with an immortal event, was performed for each agent separately to examine the association between treatment and experiment to determine if the data across the three experiments for each treatment could be combined in further analyses. Because there was no statistically significant association between experiment and treatment for the chemopreventive agents ($P = .932$) or for the antitelomerase agents ($P = .915$), the data for each treatment across three experiments were combined. Comparisons of immortalization events for the seven chemopreventive treatments and the five antitelomerase treatments were performed separately by use of two-sided chi-square tests of independence and, when significant, were followed with Tukey-type *post hoc* multiple comparison tests for proportions *(27)* to examine which treatments were statistically significantly different. The alpha level for all statistical tests was set to 0.05 and the Fisher's exact and chi-square analyses were performed by use of the SAS program (version 6.12; SAS Institute, Cary, NC). The Tukey-type *post hoc* multiple comparisons tests for proportions were programmed in Microsoft Excel, and significance was determined by consulting "Critical Values of the q Distribution" by Harter [reprinted in *(27)*, Appendix p. 57–73], where exact *P* values are not available.

Results

Concentrations of Chemopreventive Agents Used for Long-Term Study

To determine any cytotoxic effects of the chemopreventive agents, we tested a range of concentrations for each of the chemopreventive agents. Fig. 1, A, shows the dose–response of LFS-derived breast epithelial cells treated with 1 n*M*–100 μ*M* of oltipraz, DFMO, tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid compared with that of control cells. The

Table 1. Effects of chemopreventive agents on the spontaneous immortalization of Li–Fraumeni syndrome-derived breast epithelial cells

Treatment, concentration*	No. of dishes with an immortal event/No. of dishes †,‡ Experiment 1	Experiment 2	Experiment 3	*P*§	Average frequency of immortalization per 3×10^7 cells‖
Untreated	5/10	5/10	6/10		5.33×10^{-7}
Solvent	4/10	6/10	5/10		5.0×10^{-7}
Oltipraz, 10 n*M*	3/10	3/10	4/10	>.05	3.33×10^{-7}
Difluoromethylornithine, 10 n*M*	3/10	3/10	1/10	>.05	2.33×10^{-7}
Tamoxifen citrate, 10 n*M*	2/10	1/10	1/10	<.05	1.33×10^{-7}
9-*cis*-Retinoic acid, 10 n*M*	2/10	0/10	0/10	<.005	0.67×10^{-7}
13-*cis*-Retinoic acid, 1 n*M*	1/10	0/10	0/10	<.001	0.33×10^{-7}

*Cells were treated for several months with nontoxic concentrations of chemopreventive agents (*see* the "Materials and Methods" section). Three separate experiments were conducted, and the numbers of immortalization events are shown for each.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at 10^6 cells/dish.

‡The combined data had a chi-square of 37.738, 6 *df*, and a $P = .001$.

§*P* values from the Harter tables [reprinted in *(27)*] from Tukey-type multiple comparison tests for proportions comparing solvent-treated control cells. Untreated and solvent-treated controls were found to be not statistically significantly different. The solvent-treated control cells, having the lower proportion of immortalized cells, were compared with each of the other treated cells.

‖The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of 10^6 cells per dish, for a total pool size of 10×10 (or 10^7), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by 10^7, 3.0×10^{-7} *(16)*. Statistical analyses were performed using the number of immortalization events, not frequency.

Table 2. Effects of anti-telomerase agents on the spontaneous immortalization of Li–Fraumeni syndrome-derived breast epithelial cells

Treatment, concentration*	No. of dishes with an immortal event/No. of dishes †,‡ Experiment 1	Experiment 2	Experiment 3	*P*§	Average frequency of immortalization per 3×10^7 cells‖
Untreated	5/10	5/10	6/10		5.33×10^{-7}
Mismatch control, 500 n*M*	6/10	6/10	5/10		5.67×10^{-7}
Antisense RNA, 500 n*M*	2/10	0/10	0/10	<.001	0.67×10^{-7}
Vector-only control	5/10	5/10	5/10		5.0×10^{-7}
Dominant-negative hTERT	3/10	1/10	1/10	<.025	1.67×10^{-7}

*Preimmortal cells were treated for several months with nontoxic concentrations of 2′-*O*-methyl RNA or mismatch oligonucleotides. A separate set of cells was also infected with D869A-hTERT, a dominant negative-acting mutant of hTERT several population doublings before crisis (*see* the "Materials and Methods" section). Three separate experiments were conducted, and the numbers of immortalization events are shown.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at 10^6 cells/dish.

‡The combined data had a chi-square of 27.847, 4 *df*, and a $P = .001$.

§*P* values from the Harter tables [reprinted in *(27)*] from Tukey-type multiple-comparison tests for proportions comparing untreated control cells. Untreated and mismatch oligonucleotide/vector-only treated controls were found to be not statistically significantly different. The untreated control cells, having the lower proportion of immortalized cells, were compared with each of the other antitelomerase-treated cells.

‖The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of 10^6 cells per dish, for a total pool size of 10×10^6 (or 10^7), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by 10^7, 3.0×10^{-7} *(16)*. Statistical analyses were performed using the number of immortalization events, not frequency.

final concentrations of the chemopreventive agents used in these studies did not affect the growth rate of these preimmortal, noncancerous cells (Fig. 1, B). After 3 days of treatment at concentrations of 1 n*M*–1 μ*M*, the chemopreventive agents were ineffective at inhibiting telomerase activity in telomerase-positive LFS-derived breast epithelial cells that had spontaneously immortalized (data not shown). Inhibition of telomerase activity at 100 μ*M* was associated with cell growth toxicity.

Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Chemopreventive Agents on the Frequency of Spontaneous Immortalization

To determine if clinically validated chemopreventive agents have any effect on cell immortalization, we treated preimmortal LFS breast epithelial cells before undergoing crisis with oltipraz, DFMO, retinoic acid, and tamoxifen. Before any treatment, all cells lacked telomerase activity. After the cells were grown for 25 population doublings in the presence or absence of the chemopreventive agents, they underwent a period of balanced growth and death (i.e., crisis) *(16)*. The small fraction of cells that survived crisis expressed telomerase activity (Fig. 2, A) and were considered to be spontaneously immortalized because they continued to grow vigorously after crisis *(16)*. Fluctuation analysis was used to estimate the frequency of immortalization for untreated and treated breast epithelial cells. As Table 1 shows, LFS-derived breast epithelial cells treated with tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid had fewer spontaneous immortalization events compared with untreated or solvent-treated cells ($P<.05$, $P<.005$, and $P<.001$ for tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid, respectively). Long-term treatment of the cells with oltipraz and DFMO also decreased the number of spontaneous immortalization events, but these effects were not statistically significant.

Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Antitelomerase Agents on the Frequency of Spontaneous Immortalization

We treated LFS-derived breast epithelial cells with two different antitelomerase agents to determine if specifically inhibiting telomerase activity affected the frequency of spontaneous immortalization in these cells. One set of cells was transfected every 4 days over a 3-month period with an antisense RNA that was directed against the template region of human telomerase RNA. Another set of cells was infected with a retrovirus containing a dominant negative mutant of the telomerase catalytic subunit (hTERT) and then

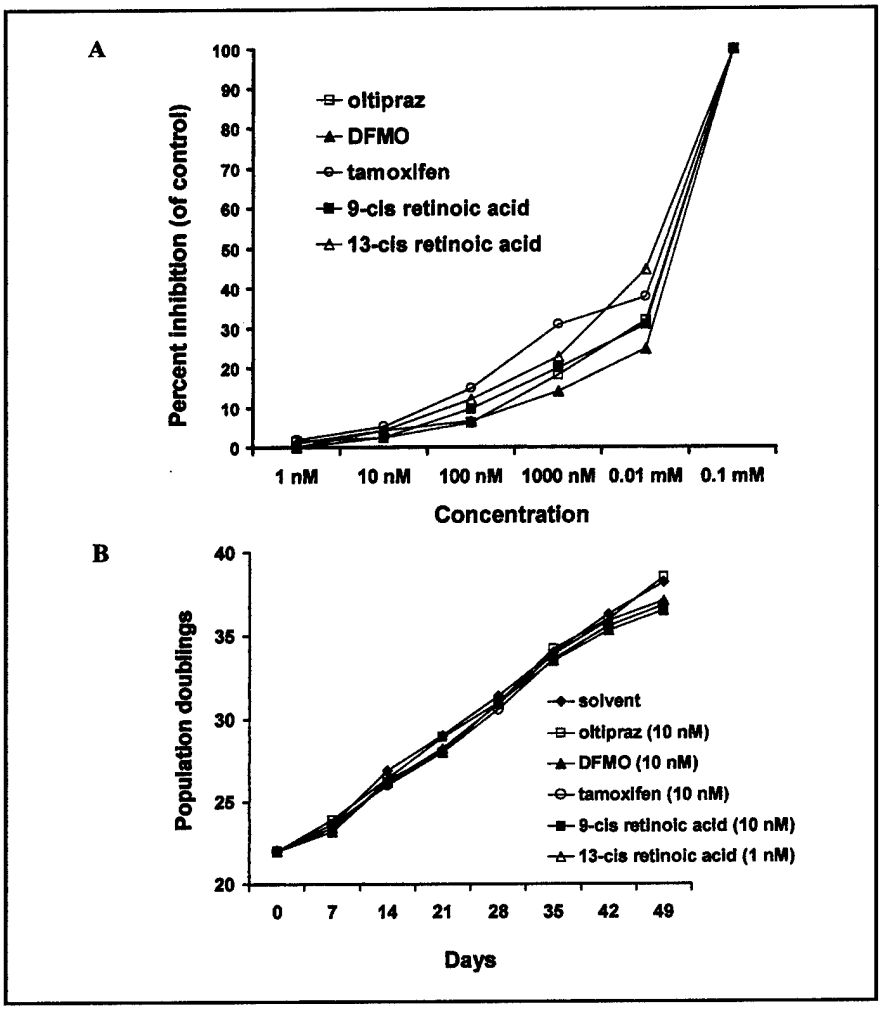

Fig. 1. Concentrations of chemopreventive agents used for long-term studies (1–10 n*M*) did not affect cell proliferation and were not toxic to the cells. **Panel A:** dose–response of growth inhibition by chemopreventive agents as shown by percent inhibition of Li–Fraumeni syndrome (LFS) breast epithelial cell number treated for 3 days with oltipraz, difluoromethylornithine (DFMO), tamoxifen, 9-*cis* retinoic acid, and 13-*cis* retinoic acid (1 n*M*–0.1 m*M*) as compared with control cells. Equal numbers of cells plated on multiple six-well dishes were treated with various doses of each chemopreventive agent or with solvent or media only (controls). After 3 days, cells were harvested and counted. The percent inhibition of cell number compared with controls was plotted. **Panel B:** growth of LFS-derived breast epithelial cells treated with nontoxic doses of the chemopreventive agents. LFS-derived breast epithelial cells were grown in culture for approximately 20 population doublings, after which they were grown in the presence of solvent or chemopreventive agents at the indicated concentrations for several weeks. Cells were split when necessary to maintain a constant cell density. Population doublings were determined by the following formula: population doublings = log(number of cells recovered at each subcultivation/number plated)/log 2. LFS-derived breast epithelial cells undergo crisis after approximately 50 population doublings in culture.

grown under conditions that selected for expression of the mutant protein. As summarized in Table 2, both antitelomerase agents decreased the absolute number of spontaneous immortalization events in the LFS-derived breast epithelial cells. The differences between untreated or mismatch- or vector-treated control cells and cells treated with the antisense oligonucleotide or the dominant-negative mutant hTERT were statistically significant ($P<.001$ and $P<.025$, respectively).

Because treatment with telomerase inhibitors did not completely prevent spontaneous immortalization of LFS-derived breast epithelial cells, we wanted to confirm whether telomerase was active in these cells after they had immortalized. The immortalized cultures derived from cells infected with the dominant-negative mutant of hTERT began to express telomerase activity, even though they were still resistant to the selectable drug resistance marker contained on the vector (data not shown). Western blot analysis detected no expression of the mutant hTERT in immortalized cells derived from cells infected with the dominant-negative mutant hTERT, confirming that the immortalized cells had lost the dominant-negative effect of the mutant hTERT because they no longer expressed the mutant protein (data not shown). The loss or decrease of expression of this mutant hTERT (and eventual reactivation of telomerase activity) within an infected cell population is not uncommon in tumor cell lines grown in continuous culture *(13)*. Therefore, the appearance of immortalized clones from cells infected with the dominant-negative mutant of hTERT was most likely due to loss of expression of the mutant hTERT and reactivation of telomerase.

To ensure that the antitelomerase antisense oligonucleotide was an effective inhibitor of telomerase activity, we infected HME cells with a retrovirus containing wild-type hTERT (HME plus hTERT cells) and then transfected them with an antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured. As shown in Fig. 2, B, transfection of HME plus hTERT cells with the antitelomerase antisense RNA effectively inhibited telomerase activity.

Discussion

This is the first demonstration that nontoxic concentrations of chemopreventive and antitelomerase agents inhibit the spontaneous immortalization of human breast epithelial cells derived from an LFS patient. Our results suggest that this approach may be a useful model system to screen for chemopreventive agents that inhibit the progression of breast cancer. These results also support the use of antitelomerase agents as an approach to prevent the activation of telomerase.

Breast epithelial cells derived from an LFS patient provide an efficient model for examining the effects of treatment on the probability of immortalization because they spontaneously immortalize at a measurable frequency of 5×10^{-7} [this study and *(16)*]. In contrast, most normal human cells, including breast epithelial cells, do not spontaneously immortalize at measurable frequencies *in vitro (28,29)*.

Immortalization is thought to be a critical, rate-limiting step in cancer progression. One mechanism by which cells immortalize is by activating telomerase. The chemopreventive agents used in this study reduced the frequency of immortalization

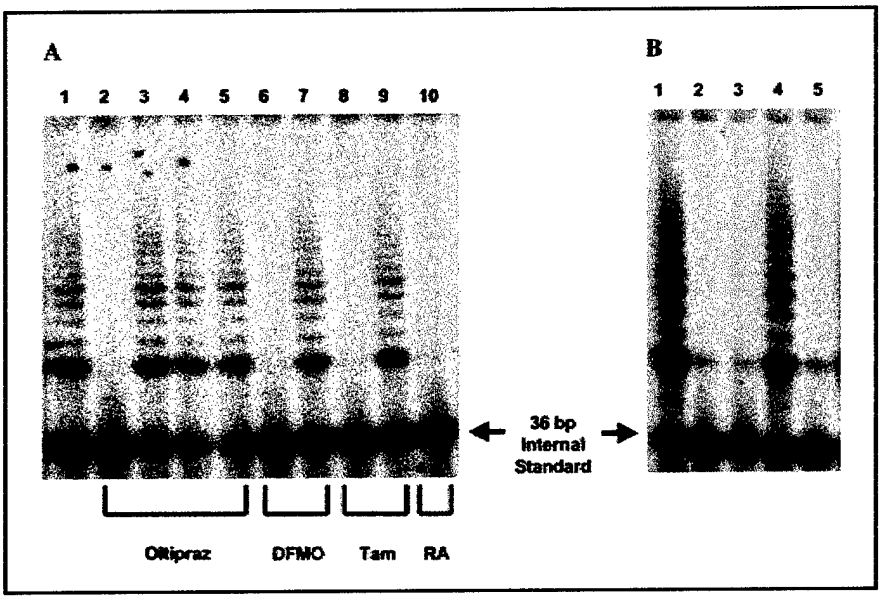

Fig. 2. Telomerase activity of treated and untreated human mammary epithelial (HME) cells. **Panel A:** cultures that immortalized after long-term treatment with chemopreventive agents expressed telomerase activity. Telomerase activity was measured for 500 HME cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based assay (*see* the "Materials and Methods" section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. **Lane 1** = untreated HME cells that have spontaneously immortalized; **lane 2** = preimmortal HME cells treated with oltipraz; **lanes 3–5** = three independent immortalization events of HME cells treated with oltipraz; **lane 6** = preimmortal HME cells treated with difluoromethylornithine (DFMO); **lane 7** = one immortalization event of HME cells treated with DFMO; **lane 8** = preimmortal HME cells treated with tamoxifen; **lane 9** = one immortalization event of HME cells treated with tamoxifen; and **lane 10** = preimmortal HME cells treated with retinoic acid. In this experiment, no immortalization events were observed in HME cells treated with retinoic acid. TAM = tamoxifen; RA = 9-*cis* retinoic acid. **Panel B:** Antisense RNA inhibits telomerase activity. Preimmortal HME cells derived from a patient with Li–Fraumeni syndrome were infected with a retrovirus containing wild-type hTERT to induce the cells to express telomerase activity. Cells expressing exogenous hTERT were then transfected with antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured for 500-cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a PCR-based assay (*see* the "Materials and Methods" section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. **Lane 1** = 500 H1299 lung carcinoma cells served as a positive control for the telomerase assay; **lane 2** = lysis buffer served as a negative control; **lane 3** = preimmortal HME cells; **lane 4** = preimmortal HME cells infected with the hTERT-containing retrovirus; and **lane 5** = preimmortal HME cells infected with the hTERT-containing retrovirus and treated with an antisense RNA oligonucleotide complementary to the template region of human telomerase RNA.

and, by some mechanism, must have prevented the ability of cells to emerge from crisis. In fact, a decrease in the frequency of immortalization was also observed for cells treated with chemopreventive agents for shorter times (only approximately 10 population doublings) before crisis. Moreover, removal of the treatments before crisis results in an immortalization frequency similar to that of untreated or mock-treated cells. These results suggest that the agents are likely to be affecting cells when they are in crisis. Whether these chemopreventive agents affect immortalization through prevention of telomerase activation is a current area of interest in our laboratory.

Since 90% of cancers exhibit telomerase activity and activation of telomerase is an early event in breast cancer progression, the development of chemoprevention strategies that specifically target telomerase is of great interest. The recent cloning of the hTERT promoter has enabled researchers to dissect the molecular mechanisms regulating transcription of hTERT *(30,31)*. Kyo et al. *(32)* showed that estrogen increases hTERT messenger RNA and activates telomerase by inducing the binding of the estrogen receptor to estrogen-response elements on the hTERT promoter. Antiestrogen therapies may, therefore, directly or indirectly affect the regulation of telomerase expression and thus prevent a step that is necessary for the progression of most cancers. One such antiestrogen drug, tamoxifen, has recently been shown to affect telomerase activity and the proliferation of breast carcinoma cell lines *(33)*. Although our results show that tamoxifen is a potent agent in the prevention of spontaneous immortalization, this effect is unlikely to involve antiestrogen activity because the HME cells used in this study do not contain the normal estrogen receptor. Tamoxifen has been shown, in some cases, to act independently of the estrogen receptor *(34)*. Whether the observed effects of tamoxifen on spontaneous immortalization of the LFS cells were indirect (i.e., through an estrogen-like nuclear receptor) remains to be determined.

Retinoids also have been shown to reduce telomerase activity, as seen after the induction of differentiation by retinoic acid and a sharp decline in cell proliferation caused by the treatment of mammary tumors with the synthetic retinoid 4-(hydroxyphenyl) retinamide *(35–38)*. These findings confirm the need to understand the molecular mechanisms by which conventional chemopreventive agents act via their respective receptors and to investigate whether they act by preventing the activation of telomerase during the progression to cancer.

Oltipraz and DFMO also decreased the frequency of immortalization, but not as much as the other chemopreventive agents used in this study. Oltipraz is an anticarcinogen that inhibits HIV-1 replication and some reverse transcriptases *(39)*. The chemopreventive properties of oltipraz have been studied in animal models of colon and lung carcinogenesis *(40,41)*. DFMO is a potent and irreversible inhibitor of the enzyme ornithine decarboxylase, which is involved in polyamine synthesis. DFMO has been reported to have chemopreventive activity in colon, skin, and breast carcinogenesis *(42,43)*. Because polyamine synthesis is necessary for cellular proliferation, DFMO may derive its chemopreventive activity from its antiproliferative properties.

Although some of the chemopreventive agents may act indirectly on telomerase, other agents are being designed specifically to target telomerase directly *(11–13,18,44–46)*. Oligonucleotides complementary to the template region of the RNA component of telomerase have been shown to inhibit telomerase activity and cell growth after long-term treatment of immortal and cancerous cell lines

(11,47,48). The other telomerase inhibitor used in this study is a cDNA that contains a point mutation in one of the conserved reverse transcriptase motifs in the human telomerase catalytic subunit, hTERT. When assembled into the telomerase ribonucleoprotein complex, this mutant subunit acts in a dominant-negative fashion to inhibit telomerase activity in tumor cell lines, shortening telomeres and inducing cell death *(12,13,21)*. Our study confirms the potential of antitelomerase agents to prevent the activation of telomerase and to inhibit spontaneous immortalization *in vitro*.

Our finding that treating cells before crisis with antitelomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells *in vitro* indicates a potential approach for the development of rational chemoprevention strategies by use of both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization described in this work offers a new intermediate end point for validating novel chemopreventive agents.

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Commentary

Telomerase and breast cancer

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Abstract

Current therapies for breast cancer include treatments that are toxic and often result in drug resistance. Telomerase, a cellular reverse transcriptase that maintains the ends of chromosomes (telomeres), is activated in the vast majority of breast cancers (over 90% of breast carcinomas) but not in normal adjacent tissues. Telomerase is thus an attractive target for both diagnosis and therapy because of its distinct pattern of expression. We address the use of telomerase in the diagnostics of breast pathology, as well as the use of telomerase inhibitors in the treatment and prevention of breast cancer.

Keywords: breast cancer, inhibitors, telomerase, telomeres, therapy

Overview of telomeres and the use of telomerase to compensate for telomere loss

Human chromosomes contain repeated TTAGGG DNA sequences at their ends (telomeres) that provide genomic stability (protect the ends from being recognized as DNA breaks needing repair) and a source of expendable DNA (a solution to the inability of the replication machinery to copy the extreme ends of chromosomes: the end replication problem) (see [1] for a review). A significant amount of truncation can occur during DNA replication without causing deleterious effects. Human telomeres progressively shorten with ongoing cell division until they reach a critical length that induces replicative senescence [2].

Recent research has led to increased knowledge of the structure and function of telomeres. Telomeres end in G-rich single-stranded 3′ overhangs, and can form a lariat structure called a t-loop. Telomere binding proteins such as TRF1 and TRF2 are thought to protect against nucleases and prevent DNA recombination or end joining [1]. Recent research has shown an increasing connection between DNA damage repair proteins (eg Mre11/Rad50/Nbs1, Ku, etc) and telomere biology in mammals. Double-strand break recognition and repair factors associated with the BRCA1-associated genome surveillance complex have, for instance, been linked to telomeres and telomere binding proteins [3]. BRCA1 or BRCA2 might also play a role in telomere structure and function.

Although other mechanisms to maintain telomere stability are possible, the mechanism for lengthening telomeres in humans is almost always by the reactivation or upregulation of telomerase [4]. Human telomerase is a protein complex consisting of a human telomerase reverse transcriptase catalytic subunit (hTERT) that uses the human telomerase RNA component (hTR) of the complex as a template for adding TTAGGG repeats to the end of the chromosome [5–7]. Telomerase is only expressed in a small number of proliferating cell types, such as germ line and somatic stem cells. Most normal human cells lack telomerase activity and their telomeres shorten with each cell division, until they enter replicative senescence. Cells that lose critical cell-cycle checkpoint functions escape this initial growth arrest and divide until they enter crisis

AZT = 3′-azido-3′-deoxythymidine; FNA = fine-needle aspirate; hTERT = human telomerase reverse transcriptase catalytic subunit; hTR = human telomerase RNA component; PCR = polymerase chain reaction; TRAP = telomeric repeat amplification protocol.

when chromosome end fusions and apoptosis occur. Cells remain in this crisis period unless a rare cell acquires a mechanism, such as telomerase expression, that can lengthen telomeres. As cells continue to proliferate, the maintenance of telomeres involves a collection of factors including telomere binding and associated proteins, the telomerase ribonucleoprotein complex and, potentially, DNA damage repair proteins. A rare cell that can maintain telomeres is then able to grow continuously (ie becomes immortal) and this is generally believed to be a critical step towards cancer progression [8].

Telomerase as a diagnostic target: incidence of telomerase in breast cancer

The development of the telomeric repeat amplification protocol (TRAP) assay led to an expansion in the ability to detect telomerase activity in human cancer cells [9]. This sensitive polymerase chain reaction (PCR)-based assay can detect as few as 1 to 10 positive cells or 0.01% in a mixed population. Although preliminary data showed 88% of all stages of breast carcinoma having positive TRAP [9], closer investigation and careful handling of initially negative samples revealed the value may be closer to 95% [10]. As reviewed by Shay and Bacchetti, 75% of breast carcinoma *in situ* lesions, 88% of ductal and lobular carcinomas, 5% of adjacent tissues, and none of the normal tissues were TRAP-positive [9]. Yashima *et al* detected a progressive increase in the mean telomerase levels with the severity of histopathological change: 14% in benign breast diseases, 92% in carcinoma *in situ* lesions, and 94% in invasive breast cancers [11]. Expression of the hTERT mRNA can also be detected using real-time quantitative reverse transcriptase-PCR, and this assay revealed a statistical link between hTERT mRNA levels and the aggressiveness of breast tumors [12]. Both this semi-automated assay and the TRAP assay provide suitable methods for breast cancer diagnosis, but should be used in conjunction with other diagnostic tools to rule out false results.

Detection of telomerase activity in preoperative specimens, such as in fine-needle aspirates (FNAs), may improve diagnostic accuracy [13,14]. FNA cytology is known to be accurate, cost effective and have minimal risk [14]; however, difficulties still occasionally occur using cytology alone. Two groups separately compared the diagnostic utility of telomerase assays of FNAs with cytology preparations [13,14]. Poremba *et al* showed that 92% of FNAs from breast cancer patients were telomerase-positive, 94% of FNAs from patients with benign breast lesions were telomerase-negative (the positive cases were all fibroadenomas), and there was a strong correlation between TRAP and histologic diagnosis of atypia [13]. Hiyama *et al* observed that all atypical or intermediate cases with detectable telomerase activity in the FNAs were found to be carcinomas after surgery [14]. Furthermore, six out of seven tumors without telomerase activity were diagnosed as benign, while one half of the cases with detectable telomerase activity, initially designated by cytology as benign, were subsequently diagnosed as cancer. Detecting telomerase activity in FNAs is thus equivalent, if not better, than detection by cytology [14], and can be used in conjunction with other diagnostic tests. Finally, tumor-derived telomerase RNA found in the serum of breast cancer patients may have implications in diagnosis and in follow-up monitoring studies [15].

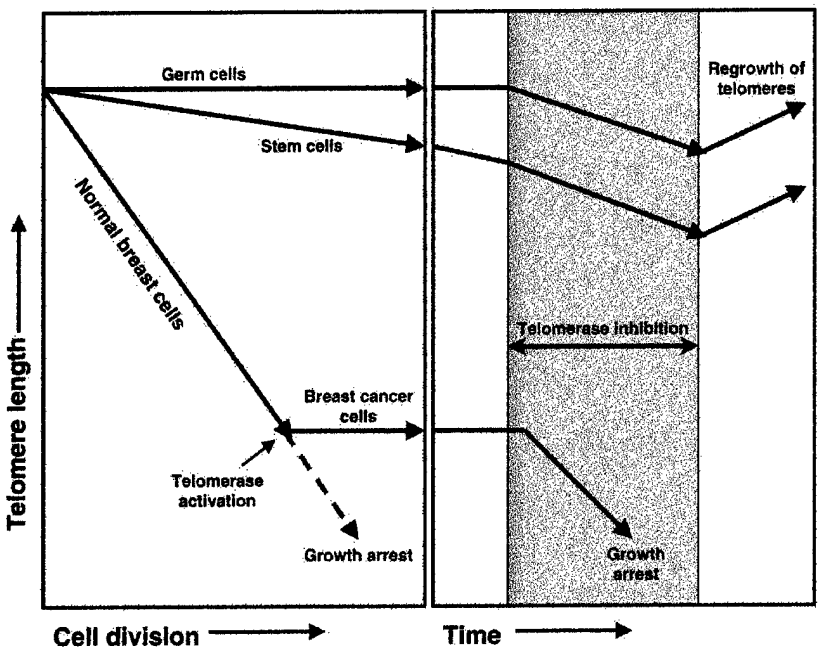
Telomerase activity and prognosis in breast cancer

With the increasing number of breast cancers detected by screening procedures, a marker is needed to stratify the risk of subsequent invasive cancer. Hoos *et al* found a significant correlation between telomerase activity and tumor size, lymph node status, and stage [16]. A significant association between telomerase-positive infiltrating breast carcinomas and lymphovascular invasion, a fundamental step in breast cancer metastasis and a predictor of survival, has also been observed, making telomerase a useful prognostic marker [17]. Clark *et al* reported, in a prognostic study involving 398 patients with lymph node-positive breast cancer, that increased telomerase activity was associated with decreased disease-free survival [18]. High telomerase activity in breast cancer is moreover associated with genetic aberrations in 3q (gain), 8q (gain), and 17p (deletion) [19]. These aberrations are common in breast cancers and involve the *hTR* (on 3q), c-*myc* (on 8q), and *p53* (on 17p) genes, all of which have been associated with telomerase regulation [19]. Understanding the link between telomerase activity and genetic changes associated with breast cancer remain an important area of research today.

Telomerase inhibition as an anticancer approach

The average telomere length in breast cancer cells is usually well below that of normal cells. This difference in telomere length coupled with the more rapid rate of cell division in cancer cells makes the inhibition of telomerase an attractive potential breast cancer therapeutic target. Treatment with telomerase inhibitors may not have the toxicity found with other chemotherapeutic agents since telomerase is absent in most somatic cells (Fig. 1). While normal, proliferating telomerase-positive stem cells may also initially be affected, their telomeres are well above the critically short length that induces a DNA damage/growth arrest mechanism. Furthermore, most stem cells are quiescent, and telomere shortening normally only occurs with cell division. Since most breast cancer cells have very short telomeres, treatment with telomerase inhibitors should lead to growth arrest and cell death.

Figure 1



Effects of telomerase inhibitors in breast cancer therapy based on reviews by Krupp *et al* [1] and White *et al* [20]. Normal breast tissues do not have telomerase activity and their telomeres progressively shorten with each cell division. When telomeres become short, cells undergo growth arrest. In rare circumstances, telomerase may be activated and a cell can become immortal, leading to accumulations of mutations and cancer. Inhibition of telomerase would lead to progressive shortening of telomeres. While normal, telomerase-competent proliferating cells, such as germ and stem cells, would be affected, their telomeres are well above the critically short length to induce a DNA damage/growth arrest mechanism. Since most breast cancer cells exhibit telomere lengths close to the critically short limit, treatment with telomerase inhibitors would lead to growth arrest and cell death. With the removal of inhibitors, telomerase would be active and telomere lengths might return to their original size.

Advances in development of telomerase inhibitors

The telomerase protein complex allows for multiple sites for inhibition. Recent research includes targeting the RNA component of the telomerase (antisense oligonucleotides, hammerhead ribozymes), inhibition of the catalytic subunit (dominant negative mutant hTERT, reverse transcriptase inhibitors), immunotherapy, and small molecule inhibitors (reviewed in [20]). It is important in most approaches to confirm that telomerase inhibitors are acting specifically in a telomere-dependent mechanism. First, telomerase inhibitors should almost always lack immediate toxicity to the cell; they should reduce telomerase activity without initially compromising cellular proliferation. Second, without telomerase activity, telomeres should progressively shorten with each cell division. Cells should ultimately die or undergo growth arrest and the time required should be related to initial telomere length. Over 100 manuscripts have been published on telomerase inhibitors but only a small number meet the presented criteria [20], and fewer still have been tested in breast cancer cells.

Antisense oligonucleotides, dominant negative mutant hTERT and reverse transcriptase inhibitors have been studied in breast cancer [20–22]. The most widely used reverse transcriptase inhibitor is 3′-azido-3′-deoxythymidine (AZT) (reviewed in [20]). Melana *et al* observed that AZT inhibited the growth of breast cancer cells and telomerase activity at lower doses than in normal breast cells [21]. Multani *et al*, however, showed that AZT reduced the telomeric signals, as detected by fluorescence *in situ* hybridization, in human MCF-7 breast carcinoma cells within 72 h [22], a time too short to be explained by the inhibition of telomerase and progressive telomere shortening. The effects of AZT, while effective in inhibiting breast cancer cell growth in these studies, may not be due to a specific telomere-dependent mechanism, and telomerase inhibition may just be a side effect [20]. Whereas further research is needed to prove the specificity of AZT, studies using both antisense oligonucleotides, such as 2′-O-methyl RNA directed against the hTR template region, and dominant negative mutant hTERT have been shown to be effective specific telomerase inhibitors in immortalized breast epithelial and breast carcinoma cells *in vitro* (reviewed in [20]).

Standard chemopreventive agents have also been investigated for a possible role in telomerase inhibition and control of breast cancer. Retinoic acid and tamoxifen have

already been shown to inhibit telomerase activity in breast cancer cells [23,24], probably as a secondary consequence of inhibition of proliferation. While these agents are known for their antiproliferative effects, not much is known on their effects on immortalization, a critical step towards breast cancer progression [8]. We have recently investigated the possibility of using these agents or specific telomerase inhibitors to prevent cellular immortalization as a breast cancer chemoprevention strategy. We found that long-term treatment of precrisis breast epithelial cells with nontoxic doses of either chemopreventive or antitelomerase agents significantly lowered the frequency of spontaneous immortalization [25]. Our studies provide a new model for the screening of novel chemopreventive agents that target cellular immortalization.

Conclusion

Telomerase is an attractive target for diagnosis and therapy since it is expressed in over 90% of breast cancer cells, while it is not expressed in most normal cells. Whereas most of the progress on the role of telomerase in breast cancer has been in diagnostics, research into telomerase inhibitors is increasing. Inhibition of telomerase *in vitro* leads to progressive telomere shortening, eventually resulting in growth arrest or cell death due to the critically short telomeres inducing a DNA damage response. Breast cancer cells with already short telomeres would be most affected by telomerase inhibitors, whereas normal stem cells with longer telomeres would be relatively resistant. The effects of telomerase inhibitors would depend on initial telomere length and rate of cell division, and it may take weeks to months to see changes in tumor size. Combining telomerase inhibitors with current therapies to reduce tumor burden may thus provide a better regimen to target breast cancer and prevent recurrence.

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TELOMERASE INHIBITORS – OLIGONUCLEOTIDE PHOSPHORAMIDATES AS POTENTIAL THERAPEUTIC AGENTS

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ABSTRACT

We have designed, synthesized, and evaluated using physical, chemical and biochemical assays various oligonucleotide N3′ → P5′ phosphoramidates, as potential telomerase inhibitors. Among the prepared compounds were 2′-deoxy, 2′-hydroxy, 2′-methoxy, 2′-ribo-fluoro, and 2′-arabino-fluoro oligonucleotide phosphoramidates, as well as novel N3′ → P5′ *thio*-phosphoramidates. The compounds demonstrated sequence specific and dose dependent activity with IC_{50} values in the sub-nM to pM concentration range.

INTRODUCTION

Human telomerase is a complex ribonucleoprotein reverse transcriptase, containing RNA (hTR) and protein (hTERT) subunits. The enzyme is believed responsible mainly for the synthesis of d-$(TTAGGG)_n$ telomeric repeats at chromosomal ends in constantly dividing cells. Telomerase activity is detected in the vast majority of immortal cell lines, as well as in various primary human tumors (1). At the same time telomerase activity is not detected in most somatic cells. Cells of constantly renewable tissues and germ line cells are the exceptions, where active telomerase is

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present. The observed differences in telomerase activity in normal versus tumor derived cells resulted in the hypothesis, that telomerase may represent a suitable target for the specific anti-cancer therapies. Following this appealing scientific rational, several classes of telomerase inhibitors were prepared and evaluated. Among the compounds tested are the small molecules (2), compounds capable of interacting with G-quadruplex structures (3), which telomeres might potentially form, dominant negative hTERT-derived proteins (4), and various types of oligonucleotides, including the phosphodiesters (5), 2–5A tethered phosphodiesters (6), phosphorothioates (7,8), 2′-O-methyl-phosphorothioate chimera (9), ribozymes (10), and PNA molecules addressed to the template region of hTR domain (7). In the recently reported crucial proof-of-principle studies, the treatment of tumor cell lines with the specific oligonucleotides or protein based telomerase inhibitors resulted in cellular senescence (4,11). The onset of cellular senescence was observed to be in direct correlation with the length of telomeres - cells with relatively short telomeres required much shorter treatments, than the ones with longer telomeres (4,11). Based on these initial encouraging results one can infer, that the inhibition of telomerase *in vivo* in cancer cells can lead to attrition of their telomeres, followed by the tumor cells death.

In this study we summarize our results outlining the design, preparation, characterization and biochemical evaluation of several different types of oligonucleotide N3′ → P5′ phosphoramidates as telomerase inhibitors. The obtained results indicate that these compounds are efficient and sequence specific telomerase inhibitors with a good potential for further development as therapeutic agents.

RESULTS AND DISCUSSIONS

The RNA component of telomerase (hTR), which is approximately 455 nucleotide long represents an attractive target for oligonucleotide based anti-telomerase agents. Among other functionally important regions, it contains a crucial eleven nucleotides long so-called template region, part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase.

First, we designed and prepared several 2′-deoxyoligonucleotide N3′ → P5′ phosphoramidates addressed to the template region of hTR in order to determine optimal oligonucleotide sequence and length requirements – see Figure 1A. The oligonucleotides were synthesized using phosphoramidite transfer methodology on ABI 392 or 394 DNA/RNA automated synthesizers (12,13).

The manufacturer recommended 1μmole DNA synthetic cycle was used, as well as the 3′-aminonucleoside-containing monomer building blocks and CPG-based solid supports, which were purchased from Annovis Inc. The compounds were analyzed and purified by ion exchange (IE) HPLC, and characterized by ^{31}P NMR, mass spectrometry, and by PAGE. The prepared oligonucleotides were

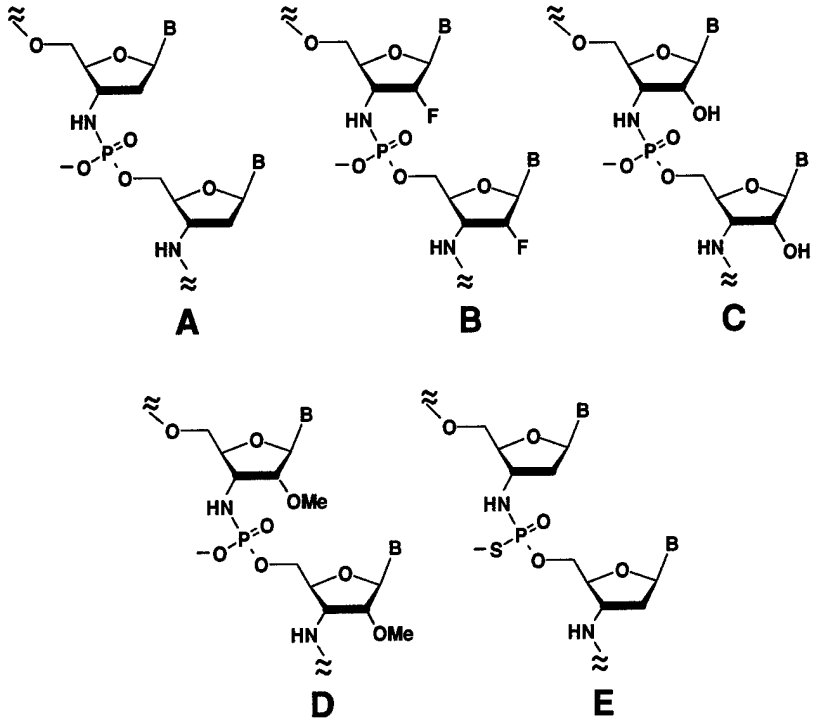

Figure 1. Structure of oligonucleotide N3′ → P5′ phosphoramidates evaluated as telomerase inhibitors.

than evaluated in biochemical assays as telomerase inhibitors, following the determination of the melting temperature (T_m) for their duplexes with the complementary RNA strand. Representative T_m data are presented in Table 3, and the obtained IC_{50} values for telomerase inhibition are summarized in Table 1.

The results indicate that IC_{50} values for the most active compounds reached the sub-nM level. Oligonucleotides with the highest anti-telomerase activity are complementary to a significant part of the hTR template region, particularly to its r-CCC segment. Moreover, anti-telomerase activity of oligonucleotides was highly sequence-dependent. Control compounds with four mismatched bases were practically inactive (more than 1000 times less active than for the fully complementary counterparts) in the assays used. These results are in a good agreement with the corresponding Tm values for the oligonucleotide duplexes: ~72°C and ~21°C, respectively, Table 3. The minimal oligonucleotide length for exerting high anti-telomerase activity is no less than six nucleotides, which also correlates well with the ability of these compounds to form stable duplexes with the RNA target – T_m

Table 1. 2′-Deoxyoligonucleotide N3′ → P5′ Phosphoramidates as Telomerase Inhibitors-Template Region

GRN#	5′-Oligonucleotide-3′	HTR[a]	IC_{50}, nM
	hTR3′-AUGCGGGAAGAGUCAAUCCCAAUCUGUU-5′[b]		
135929	GTTAGGGTTAG	56-46	0.63
135935	GTTGAG TGTAG [c]	56-46	>1000
135993	GUUAGGGUUAG	56-46	0.8
135995	GTTAGGGTTAGAC	56-44	2.4
135998	GTTAGGGTTAGACAA	56-42	1.1
135931	TTAGGG	55-50	10000
135932	TTAGGGTTAG	55-46	2.9
135939	TTAGGGTTAGGG	55-44	2.4
135970	TAGGGTTAGAC	54-44	2.0
135930	TAGGGTTAGACAA	54-42	0.8
135973	TAGGTG TGA ACAA[c]	54-42	>1000
136001	GGGTTAG	52-46	600
136003	GGGuuAG[d]	52-46	280
136000	GGGTTAGAC	52-44	5.9
136012	GGGuuAGAC[d]	52-44	<3.2
135933	AGTTAGGGTTAG	57-46	0.38
135934	CAGTTAGGGTTAG	58-46	0.47
136004	CAGTTAGGGTT	58-48	32
136995	CAGTTAGGG	58-40	32
135971	CCCTTCTCAGTT	65-54	32
136002	CGCCCTTCTCAG	67-56	40
136009	TACGCCCTTCTC	69-58	>1000
136017	CAGuuAGGGuu[d]	58-48	9
136018	GuuAGGGuu[d]	56-48	50

[a]Position of oligonucleotides on hTR; [b]sequence of hTR target, where template region is underlined; [c]mismatched nucleosides are underlined; [d]2′-fluoro-3′-aminonucleosides are depicted as low case u.

for the 2′-deoxy N3′ → P5′ phosphoramidate hexanucleotide TTAGGG is ~36°C under the assay buffer conditions.

Using oligonucleotide phosphoramidates we were also able to identify two other sites on hTR, in addition to the template hTR regions, that were susceptible to the oligonucleotide-induced telomerase inhibition-see Table 2.

The most active non-template addressed oligonucleotide was a 15-mer complementary to nucleotides 137–151 of hTR. It inhibited telomerase activity in the biochemical assays with an IC_{50} value of ~0.4 nM. Other oligonucleotide compounds addressed to the adjacent hTR regions were significantly less active, except the 20-mer GRN 137155, Table 2. It is likely, that the hybridization of the 15-mer oligomer to its hTR target site which, as predicted by molecular modeling, interferes with formation of a pseudo knot structure (14), and results in a significant structural rearrangements of entire hTR molecule. This structural alteration of hTR consequently may lead to the inactivation of the whole telomerase enzyme *via* changing the spatial positioning of hTERT catalytic site

Table 2. 2′-Deoxyoligonucleotide N3′→P5′ Phosphoramidates as Telomerase Inhibitors-Non-Template Regions

GRN#	5′-Oligonucleotide-3′	hTR	IC_{50}, nM
135940	GCTCTAGAATG	166-156	>1000
135941	GCTCTAGAATGAA	166-154	>1000
136006	GCGGCCTGAAAG	367-356	>1000
136007	TCCGTTCCTCTT	382-370	>1000
136008	GCGGGGAGCAAA	92-80	>1000
137159	GTGGAAGGCGGCAGG	151-137	0.42
137158	GCTCTAGAATGAACGGTGGAAGGCGGCAGG	166-137	1
137155	ACATTTTTTGTTTGCTCTAG	179-159	2–10

relative to the template region of hTR, or through rendering the hTR template region partially double stranded and inaccessible to telomere-like DNA primers. The 20-mer GRN 137155, Table 2, designed to hybridize to a predicted stem-loop hTR structure (14), may inhibit telomerase in a similar way. Detailed mechanism of action of non-template region addressed oligonucleotides is currently under further investigation.

Additionally, a second generation of anti-telomerase oligonucleotide N3′ → P5′ phosphoramidates with modifications at the 2′-position of the nucleoside sugar ring was designed and synthesized (12). Evaluation of these 2′-modified phosphoramidate compounds allowed for the assessment of the influence of oligonucleotide sugar-phosphate backbone structure on their activity. Several 11- to 13-mer oligonucleotides, that were isosequential to the most active 2′-deoxy - N3′ → P5′ phosphoramidate counterparts, addressed to the hTR template, were prepared. These modified phosphoramidate oligonucleotides contained 2′-fluoro, 2′-hydroxyl or 2′-O-methyl substitutions–see Figure 1B,C,D. These compounds were assembled and isolated similarly to the 2′-deoxyoligonucleotide phosphoramidates using appropriately protected 3′-amino-5′-phosphoramidite nucleoside building blocks (12). Duplex formation properties of these 2′-substituted oligonucleotide phosphoramidates with RNA strands were evaluated using thermal de-naturation experiments and the corresponding duplex T_m values are summarized in Table 3. Substitution of the oligonucleotide phosphoramidate 2′-hydrogen atom with either a 2′-hydroxyl, (resulting in generation of the natural RNA mimetic), or a 2′-O-methyl, or a 2′-fluoro group results in a noticeable stabilization of the duplexes, formed by these 2′-modified compounds with their RNA complements.

The most thermally stabile duplexes were formed by oligonucleotides containing 2′-fluoro groups. The observed stabilization of the duplexes is likely dictated by the increase in proportion of C3′-endo or N-type of nucleoside sugar puckering for the 2′-substituted oligonucleotide phosphoramidates.

The 2′-modified oligonucleotide phosphoramidates were evaluated as telomerase inhibitors in biochemical assays, and the results are summarized in Table 4. The data obtained indicate, that the activity of studied oligonucleotide

Table 3. Oligonucleotides and Melting Temperatures (T_m) of Their Duplexes with RNA Strands

Expt	5′-Oligonucleotide-3′	Type[a]	T_m,°C	ΔT_m,°C[b]
1	GTTAGGGTTAG	*d/po*	44.2	–
2	TAGGGTTAGACAA	*d/po*	45.2	–
3	GTTAGGGTTAG	*d/np*	72.1	27.9
4	TAGGGTTAGACAA	*d/np*	72.4	27.2
5	TAGG*TG*TA*AG*CAA	*d/np*	~21	−51.3[c]
6	GUUAGGGUUAG	*r/np*	80.0	35.8
7	TAGGGUUAGACAA	*r/np*	82.0	37.8
8	GU^{f}U^{f}AGGGU^{f}U^{f}AG	*2′-F/np*	84.3	40.1
9	GTTAGGGTTAG	*d/nps*	71.5	27.3
10	TAGGGTTAGACAA	*d/nps*	70.0	24.8
11	GTTAGGGTTAG	*2′-OMe/np*	78.2	34.0
12	TAGGGTTAGACAA	*2′-OMe/np*	82.3	37.1

[a]*po, np, 2′-F, nps and 2′-OMe* correspond to phosphodiester, N3′ → P5′ phosphoramidate, 2′-ribofluoro- N3′ → P5′ phosphoramidate, N3′ → P5′thiophosphoramidate, and 2′-OMe- N3′ → P5′ phosphoramidate internucleoside groups, respectively; [b]ΔT_m relative to isosequential natural phosphodiester counterparts; [c]ΔT_m relative to fully complementary compound, exp. 4, and the mismatched nucleosides are underlined.

phosphoramidates is dictated primarily by their ability to hybridize and form stable complexes with their target sites on hTR, and not by the nature of their sugar phosphate backbone. The more DNA-like 2′-deoxy phosphoramidates have similar activity to their more RNA-like 2′-substituted counterparts. Importantly, these

Table 4. Effects of Oligonucleotide Structure on Telomerase Activity

Expt#	5′-Oligonucleotide-3′	Type [a]	IC_{50},nM
1	TAGGGTTAGACAA	*d-np*	0.8
2	TAGGGTTAGACAA	*d-nps*	0.4
3	TAGGGTTAGACAA	*r-np*	0.5
4	TAGGGTTAGACAA	*r-npm*	0.5
5	TAGGGTTAGACAA-Fluor[b]	*d-np*	0.75
6	GTTAGGGTTAG-Fluor[b]	*d-np*	0.3
7	TDGGGTTDGDCDD	*d-np*	0.3
8	TAGG*TG*TA*AG*CAA[c]	*d-np*	>1000
9	TAGG*TG*TA*AG*CAA[c]	*d-nps*	110
10	CAGTTAGGGTTAG	*d-np*	0.6
11	CAGTTAGGGTTAG	*d-nps*	0.05

[a]*np, nps, npm* correspond to N3′→P5′ phosphoramidate, thiophosphoramidate and 2′-OMe-phosphoramidate oligonucleotides respectively; [b]Fluor correspond to fluorescein attached to 3′-aminogroup; [c]mismatched nucleosides are underlined.

results strongly suggest, it is unlikely that the anti-telomerase activity of various types of DNA- and RNA-like oligonucleotide phosphoramidates results from any specific, but sequence independent, high affinity interactions with only the protein component of telomerase - hTERT.

Additionally, a new class of oligonucleotide analogues – oligonucleotide N3′ → P5′ *thio*-phosphoramidates (Fig. 1E) was prepared and evaluated as telomerase inhibitors. These compounds retain high affinity to complementary RNA strands, similar to the parent phosphoramidates, yet are much more acid resistant (15). It was demonstrated before, that the oligonucleotide phosphorothioates are efficient, but highly not sequence specific inhibitors of telomerase (7,16), and that the main site of their interaction with the enzyme was likely the protein hTERT, rather than the RNA component, hTR (8). We decided to combine the attractive properties of two classes of oligonucleotides – high RNA binding affinity of the phosphoramidates and protein interactive capabilities of oligonucleotide phosphorothioates in a single molecule. The structure of oligonucleotide N3′ → P5′ phosphoramidates may allow these compounds to bind in a sequence specific manner to the targeted template region of hTR, and then create, *via* PS-groups-protein contacts, secondary stabilizing interactions with the regions of hTERT in proximity to the hTR hybridized oligonucleotide.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates were first tested in biochemical assays, and the results are summarized in Table 4. The data demonstrate high and sequence specific telomerase inhibiting activity for the template region addressed *thio*-phosphoramidate oligonucleotides. They were as active, or more active than the isosequential phosphoramidate oligonucleotides. The IC_{50} value for the most potent 13-mer reached ~50 pM, exp. 11, Table 4. Notably, mismatched control compounds were significantly less active (IC_{50} values were 100–250 folds higher), than the fully complementary oligonucleotides. At the same time, the *thio*-phosphoramidate mismatched control compounds were more active telomerase inhibitors, that the isosequential phosphoramidates, but significantly less active then the mismatched or random sequence oligonucleotide phosphorothioates. This would suggest that *thio*-phosphoramidate compounds might have some sequence – independent affinity for hTERT protein, which is in turn significantly lower, than that for the oligonucleotide phosphorothioates.

We next studied the effects of oligonucleotide N3′ → P5′ phosphoramidates on telomerase activity *in vitro* in cells. Various immortal cell lines with different levels of telomerase activity were tested, and telomerase activity was determined as a function of the oligonucleotide concentration, both in presence or absence of lipid-based uptake enhancers. The results are presented in Table 5. A typical gel profile with *in vitro* analysis of inhibition of telomerase activity by oligonucleotides is presented in Figure 2.

The collected data indicate, that oligonucleotide phosphoramidates inhibit telomerase activity in the majority of tested cell lines in a dose and sequence dependent manner. The observed IC_{50} values for telomerase inhibition by lipid

Table 5. IC_{50} Values for *In Vitro* Inhibition of Telomerase by 2′-deoxyoligonucleotide N3′ → P5′ Phosphoramidate–TAGGGTTAGACAA

	IC_{50}, μM^a Lipids	
Cell Type	(−)	(+)
U87	5	nd
293	5	0.1
ACHN	>100	0.3
Caki-1	>100	0.2
PC-3	~30	0.2
A431	>100	0.3
A549	>100	>10
HME50-5E	20	0.5
BJ-hTERT	10	0.5

[a]IC_{50} Value for MM control TAGG*TG*TA*AG*CAA with or without lipids: >100 μM.

carrier-formulated oligonucleotide phosphoramidates are significantly lower, then those for the non-formulated compounds. This may suggest relatively inefficient cellular uptake or unfavorable intracellular distribution of oligonucleotide phosphoramidates in the cell lines used. Importantly, inhibition of telomerase activity in cells by oligonucleotide phosphoramidates resulted in the concomitant reduction in the length of their telomeres, as was expected from the model for the role of telomerase in telomere maintenance.

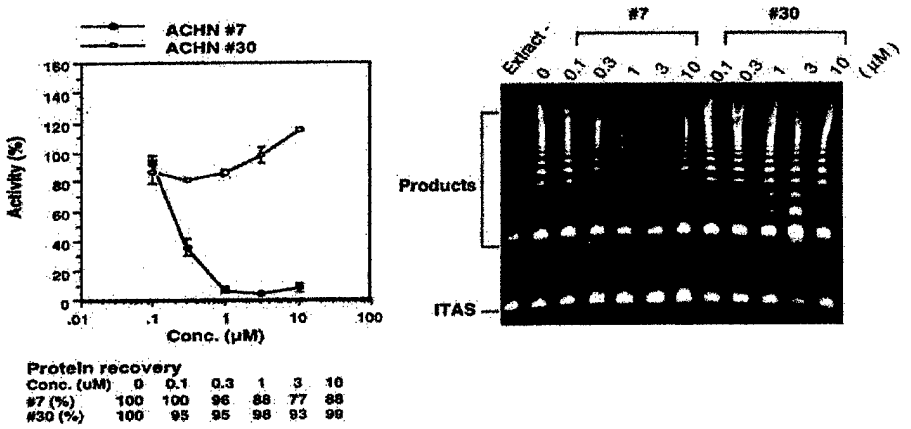

Figure 2. Analysis of telomerase inhibition in ACHN cells treated for 24 hours with Lipofectamine 2000 formulated oligonucleotide phosphoramidates by TRAP assay; #7 and #30 correspond to compounds GRN 135930 and 135973 in **Table 1**, respectively; ITAS is an internal control. Total protein recovery was measured to demonstrate the lack of acute cytotoxicity for the phosphoramidate oligonucleotides.

The obtained results demonstrate that the properly formulated oligonucleotide phosphoramidates can be used as highly efficient and specific telomerase inhibitors in cells. Further studies may identify cell lines where the used phosphoramidates will be more efficacious without the help of uptake enhancers. Moreover, *in vivo* animal models, where cellular uptake and biodistribution might be very different from the oligonucleotide behavior in cells, may serve as better indicators of the therapeutic potential for the telomerase inhibiting phosphoramidates.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates are currently under *in vitro* and *in vivo* evaluations and the results will be reported in a due course.

In summary, several classes of oligonucleotide N3′ → P5′ phosphoramidates were prepared and studied as telomerase inhibitors. The telomerase inhibiting activity of these compounds is dependent on their ability to form stable duplexes with critical segments of the telomerase RNA component hTR, and not by the nature of their sugar phosphate backbone. The most active oligonucleotides exerted IC_{50} values for sequence specific telomerase inhibition in biochemical assays in the sub-nM to pM range of concentrations, and in cells IC_{50} values were in nM to μM concentration range. The results obtained warrant further development of these compounds as efficient telomerase activity regulating agents with good therapeutic potential.

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CORRIGENDUM

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The authors wish to apologise that the name of the first author has been spelled incorrectly. The correct spelling is given above.

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Oligonucleotide N3′→P5′ phosphoramidates as efficient telomerase inhibitors

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Human telomerase is a unique reverse transcriptase that is expressed in multiple cancers, but not in the vast majority of normal cells. The enzyme is responsible for telomere protection and maintenance, and supports the proliferative immortality of cancer cells. Thus, it has been proposed that the specific inhibition of telomerase activity in tumors might have significant and beneficial therapeutic effects. To this goal we have designed, synthesized, and evaluated several oligonucleotide N3′→P5′ phosphoramidates as telomerase inhibitors. These oligonucleotides are complementary to the template region of the RNA domain of telomerase (hTR). The prepared compounds were evaluated in HME50-5E breast epithelial cells, where their effects on telomerase activity were determined using a cell-based telomerase (TRAP) assay at 24 as well as 72 h after exposure to compounds. The oligo-N3′→P5′ phosphoramidate inhibited telomerase activity in cells in the presence of the cellular up-take enhancer (FuGENE6™) in a dose- and sequence-dependent manner, with IC_{50} values of approximately 1 nM. Inhibition of telomerase activity by this compound without the lipid carrier was not efficient. However, the isosequential oligonucleotide N3′→P5′ *thio*-phosphoramidate was able to inhibit telomerase activity with or without lipid carriers at nM, or low-μM concentrations, respectively. This inhibition of telomerase activity in HME50-5E cells by the oligonucleotide *thio*-phosphoramidates was also sequence specific. Long-term treatment of the cells with 0.5 μM of FuGENE6 formulated 13-mer *thio*-phosphoramidates, fully complementary to hTR, resulted in gradual telomere shortening, followed by cellular senescence and apoptosis, as would be predicted for a telomerase inhibitor. The mismatched control compound had no effect on cell proliferation. The results suggest that the oligonucleotide N3′→P5′ phosphoramidates, and particularly *thio*-phosphoramidates, might be further developed as selective anti-telomerase reagents.
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Keywords: telomerase inhibitors; oligonucleotide; phosphoramidates

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Introduction

Human telomerase is a complex ribonucleoprotein reverse transcriptase composed of an RNA component (hTR) and protein subunit (hTERT). The enzyme is believed responsible for the synthesis of hexanucleotide d-$(TTAGGG)_n$ telomeric repeats at chromosomal ends in dividing cells, employing termini (telomeres) of DNA as primers and a segment of its own RNA as template. Telomerase activity is detected in the vast majority of immortal cell lines, as well as in various primary human tumors. At the same time telomerase activity is not detected in most mature somatic cells (Kim *et al.*, 1994). Cells of constantly renewable tissues and germ line cells are the exceptions, where active telomerase is present during periods of high proliferation. The observed differences in telomerase activity in normal versus tumor derived cells led to the hypothesis that telomerase may represent a suitable target for highly specific anti-cancer therapies. In brief, a treatment with a specific telomerase inhibitor should eventually result in telomere erosion of cancerous cells, and consequently, in their senescence and death. Following this appealing scientific rationale, several classes of telomerase inhibitors were prepared and evaluated. Among the compounds tested were small molecules (Naasani *et al.*, 1998, 1999; Bare *et al.*, 1998; Hisatake *et al.*, 1999), compounds capable of interacting with DNA G-quadruplex structures (Neidle *et al.*, 2000; Hurley *et al.*, 2000), dominant negative hTERT-derived proteins (Hahn *et al.*, 1999), antisense RNA (Feng *et al.*, 1995; Yamaguchi *et al.*, 1999), and various types of oligonucleotides, including DNA phosphodiesters (Glukhov *et al.*, 1998), 2-5A tethered phosphodiesters (Kondo *et al.*, 1998), phosphorothioates (Norton *et al.*, 1996; Matthes and Lehmann, 1999; Sharma *et al.*, 1996), phosphoramidates (Gryaznov *et al.*, 2001), 2′-O-Methyl and 2′-O-(Methoxy-ethyl) phosphorothioate chimera (Pitts and Corey, 1998; Elayadi *et al.*, 2001), ribozymes (Wan *et al.*, 1998), and PNA molecules (Norton *et al.*, 1996). The effects of DNA, PNA and 2′-modified RNA oligonucleotides reportedly were sequence specific, unlike the activity of the majority of earlier generation of phosphorothioates oligomers (Matthes and Lehman, 1999).

In the recently reported crucial proof-of-principle studies, the treatment of various tumor-derived cell

lines with oligonucleotides or protein based telomerase inhibitors resulted in cellular senescence (Herbert *et al.*, 1999; Hahn *et al.*, 1999). The onset of cellular senescence was observed to be in direct correlation with the length of telomeres; cells with relatively short telomeres required much shorter treatments than the ones with longer telomeres (Hahn *et al.*, 1999).

In this study we present our data describing evaluation of oligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates as telomerase inhibitors *in vivo* in HME50-5E cells (spontaneously immortalized human breast epithelial cell line). The obtained results indicate that these compounds are efficient and sequence-specific telomerase inhibitors with good potential for further development as promising anti-cancer agents.

Results and Discussion

The RNA component of telomerase hTR represents an attractive target for oligonucleotide-based inhibitors. Among other functionally and structurally important regions (Chen *et al.*, 2000), it contains a crucial eleven nucleotides long 'template region', part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase.

We designed and prepared several 13-mer 2′-deoxyoligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates (Figure 1), complementary to the template region of hTR. These compounds form very stable, yet sequence-specific duplexes with complementary RNA strands, while remaining highly resistant to hydrolysis by cellular nucleases (Gryaznov, 1999; Pongracz and Gryaznov, 1999). Thus, the melting temperature (T_m) for the complexes formed by the N3′→P5′ phosphoramidate oligonucleotides np-TAGGGTTAGACAA (1) and np-CAGTTAGGGTTAG (2) with RNA was 72.4°C and 73.0°C respectively, under close to physiological conditions (in PBS

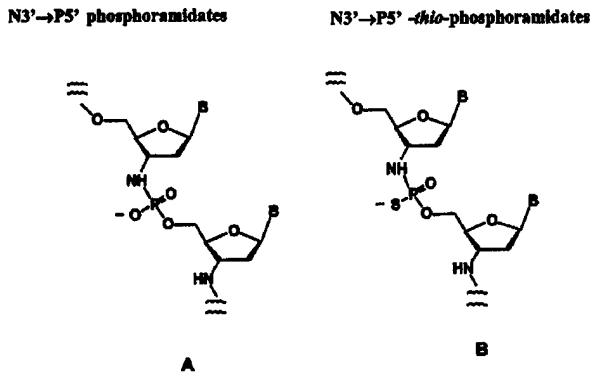

Figure 1 General structure of oligonucleotide N3′→P5′ phosphoramidates – (**a**), and N3′→P5′-*thio*-phosphoramidates – (**b**)

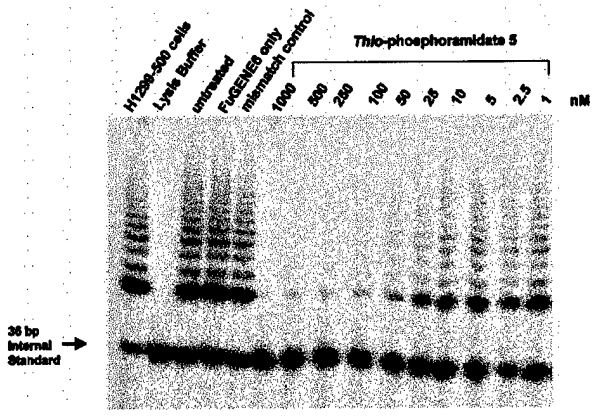

Figure 2 Dose-dependent inhibition activity by *thio*-phosphoramidate oligomer 5 delivered into HME50-5E cells by using FuGENE6 lipid, as measured 24 h post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TRAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Five hundred cell equivalents from H1299 cells served as positive control for the assay. Lysis buffer alone served as a negative control

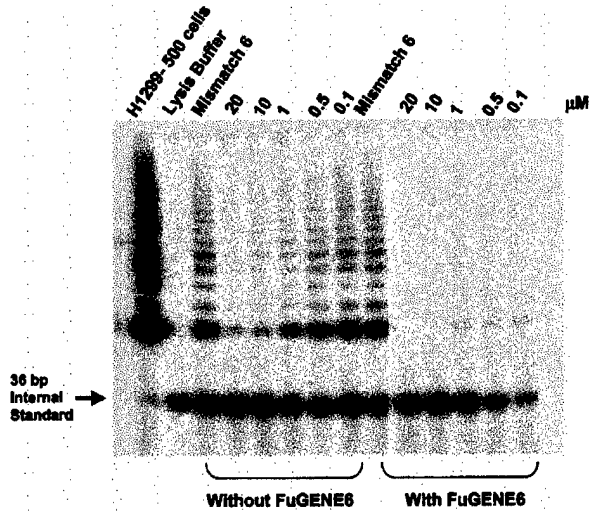

Figure 3 Dose-dependent inhibition activity by *thio*-phosphoramidate oligomer 4 delivered into HME50-5E cells with or without the use of FuGENE6 lipid, as measured 24 h post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TEAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Five hundred cell equivalents from H1299 cells served as positive control for the assay. Lysis buffer alone served as a negative control

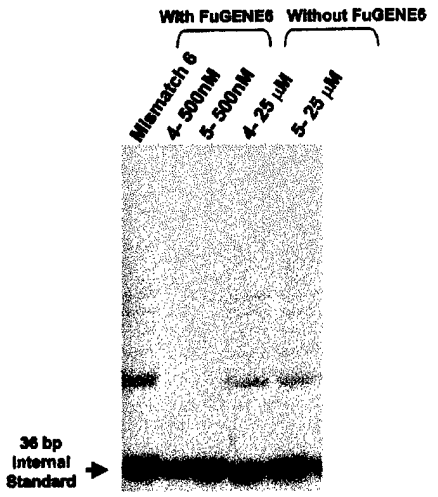

Figure 4 Inhibition of telomerase activity during the long-term treatment by *thio*-phosphoramidates 4 and 5 delivered into HME50-5E cells with or without the use of FuGENE6 lipid, as measured 3 days post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TRAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase

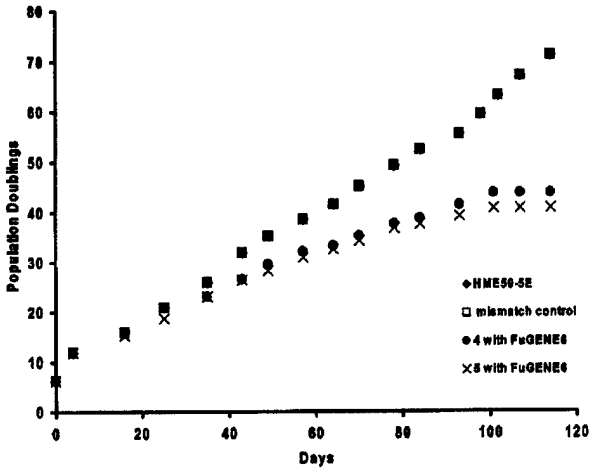

Figure 5 Effects of match 4, 5, or mismatch 6 *thio*-phosphoramidate oligonucleotides (formulated with FuGENE6) on HME50-5E cell growth during long-term treatment experiments. The concentration of all oligonucleotide was 500 nM

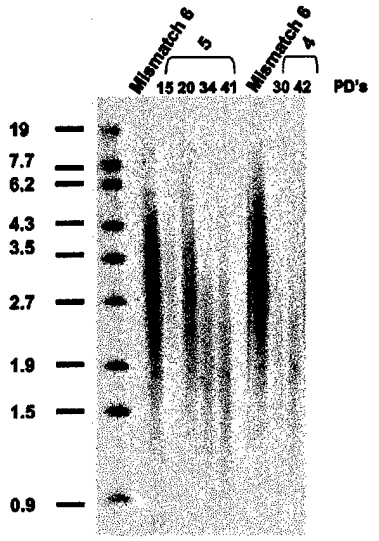

Figure 6 Measurement of telomere restriction fragment length (TRF) in HME50-5E cells treated (long-term, see Figure 5) with match *thio*-phosphoramidate oligomers 4, 5, or mismatch 6. Equivalent amounts of chromosomal DNA were loaded in each lane. The TRF lengths are expressed as kilobase pairs

buffer). The high thermodynamic stability of complexes formed by these oligonucleotides with RNA, we believe, is crucial for their ability to recognize and bind, via duplex formation to hTR, and consequently to inhibit the enzyme. Another important factor is the position of the targeted site on hTR. It is also important to note that the mismatch control oligonucleotide np-TAG**GT**GTA**AG**CAA (mismatched nucleosides are in bold and underlined) (3), having the same nucleoside composition, formed much less stable duplexes with the same RNA strand with T_m value of $\sim$21°C.

The isosequential N3′→P5′ *thio*-phosphoramidate oligonucleotides (NPS) formed duplexes with RNA and demonstrated similar thermal stability – T_m values for nps-TAGGGTTAGACAA, nps-CAGTTAGGGTTAG, and nps-TAG**GT**GTA**AG**CAA were 70.0°C, 70.5°C, and <20°C, respectively. These compounds were designated as 4, 5, and 6, respectively.

Following the duplex stability studies, the ability of oligonucleotide N3′→P5′ phosphoramidates to inhibit telomerase activity was evaluated in HME50-5E cells. The cells were exposed to varying concentrations of the compounds in the absence or presence of cationic lipids FuGENE6™. Telomerase activity was determined as a function of the oligonucleotide treatment using a cell-based TRAP assay (Kim *et al.*, 1994) 24 h after exposure to the compounds. The obtained data indicate that hTR complementary oligonucleotide phosphoramidates (NP's) very efficiently inhibited telomerase activity in cells in the presence of the cellular uptake enhancer, with IC_{50} value of $\sim$0.5–1 μM, whereas a mismatched control compound had no effect. In the absence of lipid carrier the anti-telomerase activity of the NP compound was noticeably lower, with IC_{50} value of approximately 20 μM, with mismatched control oligonucleotide not being active at concentrations up to 100 μM (data not shown). Inefficient cellular up-take or intracellular distribution, (such as endosomal sequestration) of lipid un-formulated NP oligonucleotides is likely a key factor reducing the activity of these compounds in cells in the absence of lipid carriers.

We next analysed the effects of telomerase-addressed *thio*-phosphoramidate (NPS) oligonucleotides in HME50-5E cells. The experiments were conducted

similarly to the ones with their NP counterparts. The obtained data demonstrate that NPS oligonucleotides were significantly more efficient telomerase inhibitors, both with and without cellular up-take enhancers, than the parent phosphoramidate compounds. Thus, the IC_{50} values for telomerase inhibition by oligonucleotides 4 and 5 were ~ 5 nM and ~ 0.5 nM respectively, both in presence of FuGENE6™. At the same time mismatched control NPS compound 6 was not active, with an IC_{50} value much higher than 1000 nM. Importantly, even in absence of lipid carrier NPS oligonucleotides were able to inhibit telomerase in cells; IC_{50} value for compound 4 was approximately 0.5 μM, whereas for the control oligonucleotide 6 it was more than 20 μM. Characteristic gel profiles for cell-based TRAP analysis of telomerase inhibition by NPS oligonucleotides are presented in Figures 2 and 3.

Furthermore, the effects of telomerase-specific NPS oligonucleotides on cellular proliferation were tested in long-term assays. HME50-5E cells were treated with NPS compounds 4, 5 and 6 in the presence or absence of FuGENE6™ at concentrations of 0.5 μM or 25 μM, respectively. The oligonucleotides were added to the cells on every fourth-to-fifth day. Telomerase activity, as well as cellular proliferation (population doublings) was measured during the course of the experiment. The telomerase activity in cells transfected with FuGENE6™ formulated 4 and 5 oligonucleotides was reduced to practically undetectable levels, as determined 3 days post addition of the compounds. Mismatched control 6 again did not affect the enzyme at this concentration (Figure 4). In contrast, un-formulated oligonucleotides exerted a much weaker anti-telomerase effect, even at 50-fold higher doses (Figure 4).

Inhibition of telomerase activity by NPS oligonucleotides 4 and 5 resulted in reduction of the cellular proliferation rate, culminating in the onset of cellular senescence at approximately day 100, followed by massive apoptosis at day 115 of the experiment. At the same time the cells, which were transfected with the mismatched control compound 6, proliferated at the same rate as the untreated counterparts (Figure 5). Moreover, inhibition of telomerase activity by NPS oligonucleotides was accompanied by concomitant reduction in the average telomere length, from ~ 3 kb to ~ 1.9 kb, at the time of senescence, as determined by TRF analysis (Figure 6). The length of the shortest telomeric fragments was reduced from ~ 1.9 kb to ~ 0.9 kb. The un-formulated (without FuGENE6™) NPS oligonucleotides did not significantly change either cellular proliferation or the length of telomeres in comparison with untreated cells (data not shown). The dramatic difference in the observed effects of the NPS oligonucleotides with versus without lipids is likely due to the significantly lower intracellular or intranuclear concentration of oligonucleotides available for interaction with telomerase in the absence of lipid carrier such as FuGENE6™, resulting in a relatively low level of telomerase inhibition in the cells used. Consequently, we would like to suggest that very high, almost complete level of telomerase inhibition, as was achieved only with lipid formulated NPS oligonucleotides 4 and 5 (see Figure 4), is crucial for induction of telomere shortening and the onset of cellular senescence. In HME50-5E cells, lower levels of telomerase inhibition by un-formulated NPS compounds apparently are not sufficient to cause telomere length reduction.

In summary, oligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates were designed, prepared, and studied as telomerase inhibitors in cells. The telomerase inhibiting activity of these compounds is highly sequence-specific and dependent on their ability to form stable duplexes with the template region of hTR. The oligonucleotide *thio*-phosphoramidates were significantly more potent telomerase inhibitors than the cognate phosphoramidate compounds, both with or without a cellular up-take enhancer. The NPS oligonucleotides inhibit telomerase activity in immortal HME50-5E cells with IC_{50} values of 0.5–5 nM and 0.5-1 μM with and without lipid carrier FuGENE6™, respectively. Mismatched control oligonucleotides were completely ineffective at the concentrations used. Inhibition of telomerase activity in these cells eventually resulted in a gradual reduction in the length of telomeres, followed by the onset of cellular senescence and apoptosis.

The results obtained warrant further and thorough evaluation of these compounds in *in vivo* and *in vivo* systems as efficient and specific anti-telomerase agents with good therapeutic anti-cancer potential.

Acknowledgments
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$p16^{INK4a}$ inactivation is not required to immortalize human mammary epithelial cells

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Using standard culture conditions, primary human mammary epithelial cells (HMECs) undergo a premature, transient growth arrest termed M0 (mortality stage 0) after 10–15 population doublings *in vitro*. It has been reported that emergence from this growth arrest by the abrogation of $p16^{INK4a}$, a cyclin-dependent kinase inhibitor, and expression of the catalytic component of human telomerase (hTERT) are necessary for HMEC immortalization. Here we show that primary HMECs, grown on feeder layers, do not undergo this growth arrest and can be immortalized without abrogating p16. These findings support the concept that the so-called M0 stage represents a cell culture stress-induced growth arrest and that hTERT is sufficient to immortalize HMECs when cultured under adequate conditions.
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Keywords: telomerase; senescence; culture; methylation; growth arrest

Previous reports have shown that under some culture conditions human mammary epithelial cells (HMECs) undergo a self-selection or M0 stage after an initial growth period of approximately 10–15 population doublings (Romanov *et al.*, 2001; Kiyono *et al.*, 1998; Brenner *et al.*, 1998; Huschtscha *et al.*, 1998). Emergence from this transient growth plateau is correlated with loss of p16 expression (Romanov *et al.*, 2001; Kiyono *et al.*, 1998; Brenner *et al.*, 1998; Huschtscha *et al.*, 1998). It has also been claimed that loss of p16 expression, in addition to the expression of the catalytic component of human telomerase (hTERT), is required for the immortalization of HMECs (Kiyono *et al.*, 1998). The premature growth arrest and loss of p16 in several epithelial cell types are due to inadequate growth conditions that can be overcome by the use of feeder layers (Ramirez *et al.*, 2001; Shay and Wright, 2002). We therefore sought to determine whether HMECs, under adequate growth conditions, could be immortalized with human telomerase without the need to abrogate p16.

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We first transferred the early passage HMECs (derived from organoid cultures) from plastic culture dishes to dishes with feeder layers (mitomycin-treated 3T3 cells) to prevent a p16-induced growth arrest. HMECs grown on feeder layers did not exhibit an early growth slow down while HMECs grown on plastic showed a typical plateau phase associated with 'self-selection' (Figure 1, Romanov *et al.*, 2001; Kiyono *et al.*, 1998; Brenner *et al.*, 1998; Huschtscha *et al.*, 1998; Ramirez *et al.*, 2001). Thus, M0 is not senescence (Romanov *et al.*, 2001), but a culture-condition dependent growth arrest and selection stage from which HMECs can emerge at a high frequency.

We next tested whether the catalytic component of human telomerase (hTERT) was able to immortalize human mammary epithelial cells while growing on feeder layers. HMECs infected with hTERT-expressing retrovirus vectors showed positive telomerase activity as shown by the TRAP assay (Figure 2a). Continuous growth of the HME+hTERT cells on feeder layers allowed for the direct immortalization of these cells using standard HMEC growth conditions plus 1% serum (Figure 2b). The cells have now divided more than 120 times, more than three times that of controls, and are continuing to divide at normal rates without any signs of slowing down. Continued culture of the vector only and uninfected controls did not result in spontaneous resumption of growth. Serum has been described as one factor inducing the differentiation of basal to luminal cells (Ethier *et al.*, 1993). The protein p63 is a marker for basal cells (DiRenzo *et al.*, 2002). Figure 2c shows that the low concentration of serum needed to support the feeder layers was not sufficient to cause this transition and that the immortalized cells retained the basal cell marker. The immortalized cells were also positive for other basal cell markers, such as the keratins 14 and 18 (data not shown). Cytogenetic analyses showed that the HME+hTERT cells grown on feeder layers retained a normal karyotype and did not exhibit the multiple abnormalities found following self-selection (Romanov *et al.*, 2001).

p16 protein levels did not increase in late passage HMECs grown on feeder layers nor did the levels change with the expression of hTERT (Figure 3). The $p14^{ARF}$ protein was not detectable in HMECs (Figure 3). Hyper- and hypo-phosphorylated forms of the retinoblastoma protein (Rb) were detected in the HME+hTERT cells grown on feeder layers compared

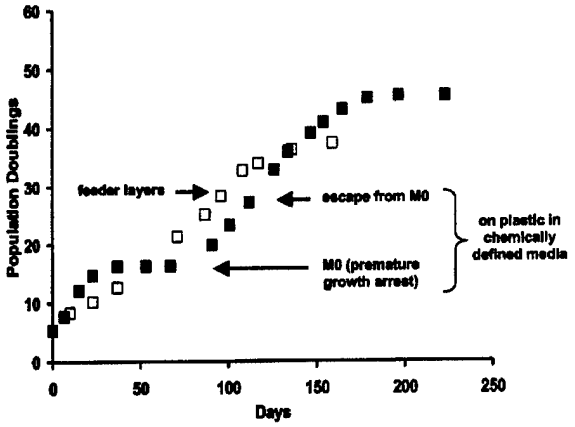

Figure 1 HMEC growth curves on plastic versus feeder layers. HMECs grown on feeder layers (open squares) grow for 35–40 population doublings before slowing down while HMECs grown on plastic (filled squares) experienced a growth plateau termed self-selection or 'M0' at 10–15 population doublings, escaped from M0 and then grew an additional 30–35 population doublings. HMECs were derived from a 33-year-old patient's breast tissue with no history of breast cancer. Cells for primary culture were obtained and co-cultured with mitomycin-treated 3T3 feeder layers as described previously (Ramirez *et al.*, 2001). Briefly, 2×10^4 cells/cm^2 mitomycin-treated 3T3 cells were plated as feeder layers and allowed to attach before plating the HMECs at 10^4 cells/cm^2. Cells were fed every other day and split when the HMECs were 80% confluent (feeder layer cells occupied 20% of the dish). To separate feeder layer cells from HMECs, feeder layer cells were detached and discarded from the culture dish by extensive pipetting with 0.02% EDTA. After ensuring under a light microscope that the feeder layer cells were removed from the dish, the HMECs were split as usual with trypsin/EDTA

to Hela cells (a cervical carcinoma cell line), which showed the hypophosphorylated form only, and MCF-7 cells (a breast carcinoma cell line), which showed both hyper- and hypo-phosphorylated forms (Figure 3).

We examined whether the immortalized HMECs grown on feeder layers retained p16 protein that responded to cellular stress. First, we transferred HME+hTERT cells grown on feeder layers to plastic dishes in chemically defined media, reasoning that the removal of the mesenchymal (feeder layer) cell contact or secreted growth factors could potentially activate the p16-induced cellular stress response. A strong upregulation of p16 five to seven days after shifting to plastic culture dishes was observed as well as a growth arrest of the entire HME cell population (Figure 4). We also infected the hTERT immortalized HMECs growing on feeder layers with a retroviral vector encoding the Harvey Ras sarcoma viral oncogene homologue (H-RasGV12). The addition of Ras resulted in increased p16 levels (after 5–7 days) and ultimately a growth arrest of the entire HME cell population (Figure 4). While Ras is known to effect the expression of other proteins, this indicates that p16 is functional and likely to be involved in the growth arrest (Serrano *et al.*, 1997; Robles and Adami, 1998).

Post-M0 selection cells frequently contain a methylated p16, and treatment with the methylation inhibitor, 5-aza-2-deoxycytidine, results in the activation of p16 protein expression and growth arrest (Brenner *et al.*, 1998; Figure 4). In contrast, the addition of 5-aza-2-deoxycytidine for 72 h to HME+hTERT cells grown on feeder layers did not increase the amount of p16, indicating that p16 was not methylated or hemi-methylated (Figure 4). All three of these results confirm that HME+hTERT cells grown on feeder layers retained p16 protein that responds to cellular stress.

Here we show that, under adequate growth conditions, telomerase activity alone can immortalize HMECs without the inactivation of p16. Culturing HMECs on plastic culture dishes induces a self-selection or M0 stage mediated by increased p16 levels and leading to a premature (telomere-independent) growth arrest (Romanov *et al.*, 2001; Kiyono *et al.*, 1998; Brenner *et al.*, 1998; Huschtscha *et al.*, 1998). Romanov *et al.* (2001) stated that this plateau exhibited characteristics of senescence but was not a permanent barrier to further growth. These authors redefined this stage, previously referred to as M0 (from which cells emerge spontaneously at a high frequency) as senescence (Romanov *et al.*, 2001). Our results demonstrate that this arrest cannot be considered senescence since it does not occur when cells are provided with a more favorable environment. Culturing on feeder layers provides adequate growth conditions so that HMECs do not upregulate p16 and thus can be immortalized with hTERT alone. Ongoing data with two other primary HMEC strains indicate the lack of p16 abrogation on feeder layers is a generalized phenomenon and not specific to one HMEC strain. These findings support the hypothesis for a stress-induced abrogation of p16 of HMECs growing on plastic culture dishes, but more importantly indicate that loss of p16 is not required for HMEC immortalization. Inactivation of p16 is clearly occurring in many tumors, and it may be silenced because it contributes to a checkpoint arrest of invasive cells that migrate into an inappropriate environment. However, to date there is no evidence supporting an independent role for p16 in the regulation of replicative aging. In the absence of *in vivo* data suggesting other counting mechanisms, we propose the adoption of the functional definition that culture conditions are adequate if they permit cells to reach a telomere-based replicative senescence that can be bypassed by the expression of telomerase (Shay and Wright, 2002).

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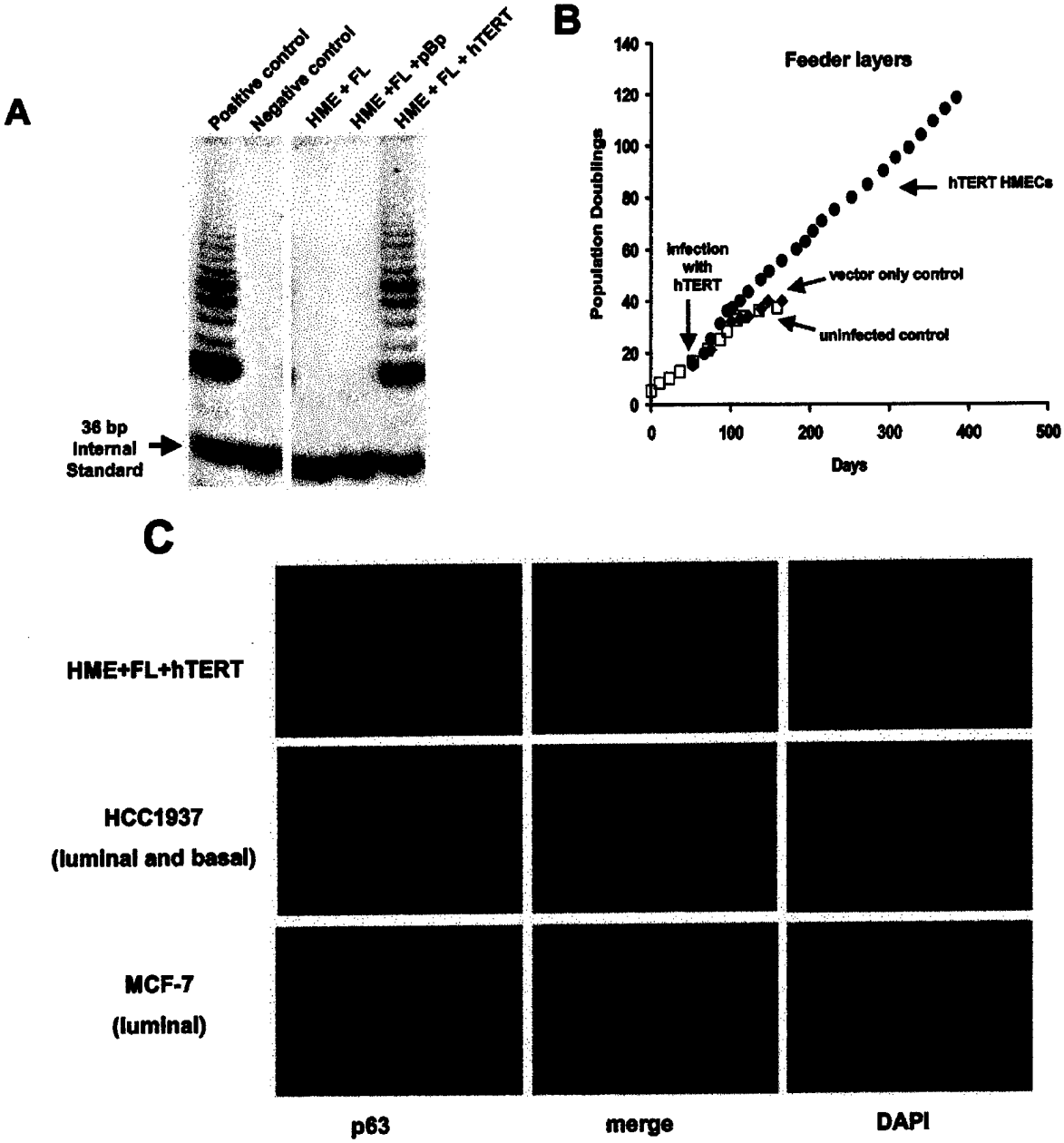

Figure 2 HMECs grown on feeder layers are immortalized by telomerase and retain basal epithelial characteristics. (**a**) TRAP analysis revealed telomerase activity in the hTERT infected HME cells grown on feeder (FL) layers (HME + FL + hTERT) compared to uninfected (HME + FL) or vector (pBp) control cells (HME + FL + pBp). HMECs at approximately 30–50% confluency growing in 100 mm plates in the presence of puromycin-resistant feeder layers were infected with pBABE alone or pBABE containing hTERT (Ouellette *et al*., 1999) in the presence of 4 μg/ml of Polybrene (Sigma) for 10–12 h. Seventy-two hours later, the cells were selected with 350 ng/ml of puromycin. Telomerase activity was measured using the TRAP-eze Telomerase Detection kit (Intergen) and established protocols (Piatyszek *et al*., 1995; Wright *et al*., 1995). A telomerase expressing lung carcinoma cell line and lysis buffer only served as positive and negative controls, respectively. (**b**) HMECs grown on feeder layers infected with hTERT (filled circles) grow indefinitely while HMECs grown on feeder layers alone (open squares) or infected with vector only (filled diamonds) senesce after 35–40 population doublings. (**c**) HME + FL + hTERT cells express the nuclear basal marker p63 while a luminal breast carcinoma cell line (MCF-7) does not. The BRCA1-mutated cell line (HCC1937) showed an intermediate phenotype between luminal and basal. HMECs grown on cover slips overnight were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 before blocking with 3% BSA. Staining was performed with a p63 antibody (Santa Cruz) diluted in PBS with 0.5% sodium azide and an Alexa-Fluor 568-conjugated secondary antibody (Molecular Probes). The coverslips were mounted with Vectashield containing DAPI (Vector Laboratories) and cells were examined using a Zeiss fluorescent microscope

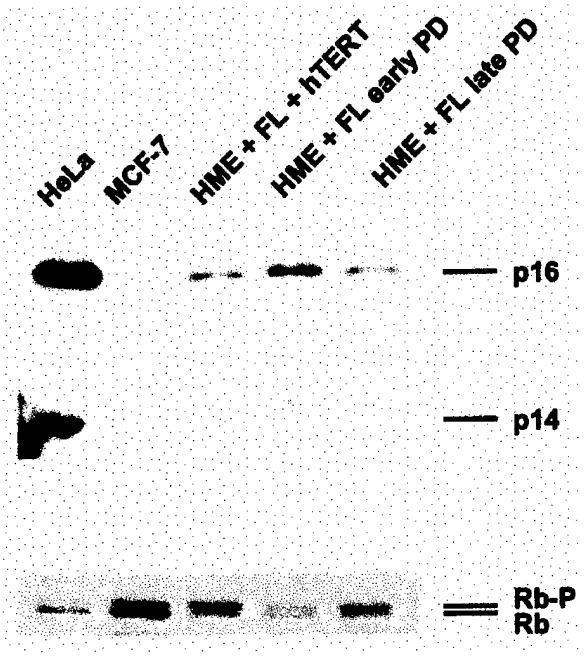

Figure 3 p16, p14, and Rb protein expression in HMECs grown on feeder layers. Logarithmically growing cells were lysed in 2% sodium dodecyl sulfate (SDS) in 50 mM Tris-HCl and total protein concentration determined using the BCA assay (Pierce). Fifty micrograms of each sample were electrophoresed on a 15% SDS polyacrylamide gel and transferred to Immobilon-P PVDF membrane (Millipore). The membrane was blocked in 5% nonfat dry milk, incubated with 2 μg/ml $p16^{INK4a}$ (PharMingen), p14 (Novus Biologicals), or Rb (Santa Cruz) primary antibodies overnight at 4°C, washed with PBS/0.1% Tween 20, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham) at a dilution of 1:5000. After washing, specific protein bands were detected using a chemiluminescent substrate (Super-Signal Substrate, Pierce). Equal loading of samples and efficient transfer was verified with Ponceau S staining (Sigma Biochemicals). A cervical carcinoma cell line (Hela, expressing p16) and a breast carcinoma cell line (MCF-7, lacking p16) served as controls

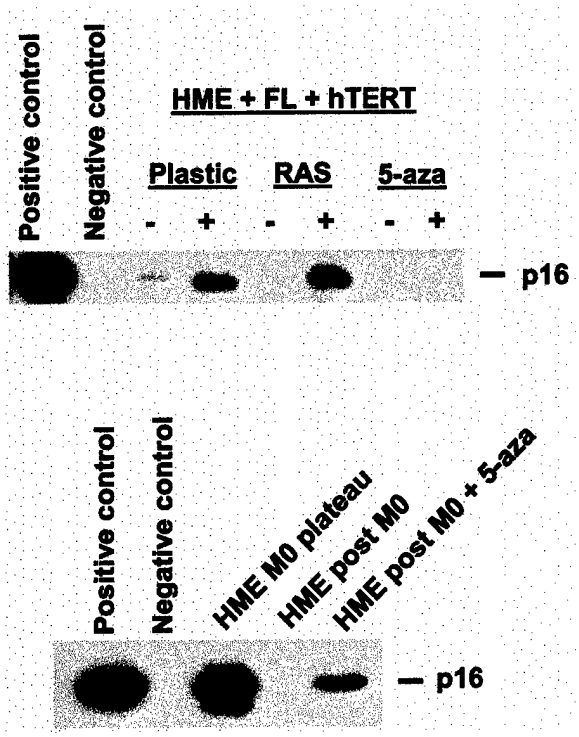

Figure 4 Immortalized HMECs grown on feeder layers retain p16 protein that can respond to cellular stresses. Western analyses showed that HME+hTERT cells grown on feeder layers (HME+FL+hTERT) transferred to plastic dishes increased p16 protein levels. HME+FL+hTERT cells infected with RAS (Morales *et al.*, 1999) also showed increased p16 protein levels compared to uninfected cells. The addition of 5-aza-2-deoxycytidine (5-aza) to HME+FL+hTERT cells did not increase the amount of p16, indicating that p16 was not hemi-methylated. Western analyses were performed as described in Figure 3. The increase in p16 protein levels by cellular stresses are comparable to the levels elicited by culturing HMECs on plastic at the M0 growth arrest plateau and when post-M0 HMECs are treated with 5-aza-2-deoxycytidine. A cervical carcinoma cell line (Hela, expressing p16) served as an internal positive control so that protein expression could be interpreted relative to the positive control

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A Peroxisome Proliferator-activated Receptor-γ Agonist and the p53 Rescue Drug CP-31398 Inhibit the Spontaneous Immortalization of Breast Epithelial Cells[1]

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ABSTRACT

Cell immortalization is a critical and rate-limiting step in cancer progression. Agents that inhibit cell immortalization may have utility for novel molecular chemopreventive strategies. Preimmortal breast epithelial cells derived from a patient with the Li-Fraumeni Syndrome (LFS) can spontaneously immortalize *in vitro* at a measurable and reproducible frequency. In the present study, these cells were treated *in vitro* with low (nM) concentrations of potential and otherwise clinically validated chemopreventive agents, including several nonsteroidal anti-inflammatory drugs, rosiglitazone maleate, and the p53 rescue drug CP-31398. Rosiglitazone maleate ($P < 0.05$) and CP-31398 ($P < 0.05$) significantly inhibited the frequency of spontaneous immortalization of LFS breast epithelial cells compared with untreated controls. Nonsteroidal anti-inflammatory drugs, including specific cyclooxengenase-2 inhibitors, only moderately inhibited the spontaneous immortalization of preimmortal LFS breast epithelial cells. The significant effects of the p53 rescue drug CP-31398 correlated with the increase in cellular death induced by telomere shortening-induced DNA damage signals, including increases in p53 and p21 protein levels. Because immortalization is one step in cancer progression, these studies show the potential usefulness of a cell-based model system to screen the effects of known and potentially novel chemopreventive agents, using cell immortalization as an end point.

INTRODUCTION

Spontaneous immortalization of human cells *in vitro* is an extremely rare event, requiring mutations in several genes and cellular pathways normally involved in cellular senescence (1–3). Normal cells have a limited life span and undergo replicative senescence, in which cells cease to proliferate (4–6). Senescence occurs when cells contain at least some critically short telomeres. The maintenance of functional telomeres (consisting of TTAGGG repeats in humans) is essential for protecting the chromosome ends from being recognized as damaged DNA (7). Because of the end replication problem, where lagging strand synthesis cannot copy all of the way to the very end of the lagging strand, almost all normal human cells gradually lose telomeric DNA as they age (8–10). This growth arrest is believed to be triggered by a DNA damage repair signaling program (11). Cells that lose critical cell cycle checkpoint functions escape this initial growth arrest and divide until they enter crisis, when telomere lengths become extremely short, and chromosome end fusions and apoptosis occur (12, 13). Immortal cells escape crisis, a period of balanced cell growth and death usually followed by a decrease in the total number of surviving cells, when telomerase or another mechanism to maintain telomere stability is activated (14, 15). Once telomerase is activated, it preferentially elongates the critically short telomeres, stabilizes telomere lengths, and permits continued cell division. This hypothesis is supported by the observation that ectopically introduced telomerase activity can extend telomeres and indefinitely prolong cellular life span (16, 17). Over 90% of breast carcinomas have been shown to contain telomerase activity, whereas adjacent normal tissues do not express telomerase activity (18–20).

Although normal human breast epithelial cells *in vitro* rarely spontaneously immortalize, LFS-derived[3] (p53 +/−, telomerase silent) breast epithelial cells have been shown to spontaneously immortalize at a relatively high and reproducible frequency of $\sim 5 \times 10^{-7}$ (21, 22). Telomerase inhibitors have been shown to inhibit the spontaneous immortalization of LFS-derived breast epithelial cells by preventing the activation of telomerase (22). The fact that the LFS-derived breast epithelial cells can reproducibly and spontaneously immortalize allows investigations into the effects of different agents on the immortalization frequency *in vitro*. In the present study, we surveyed a panel of potentially novel chemopreventive agents, including several NSAIDs, a peroxisome PPARγ agonist, and the p53 rescue drug CP-31398, for their effects on the spontaneous immortalization of LFS-derived breast epithelial cells. Here, we show that treatment of LFS-derived breast epithelial cells just before crisis with low (nM range), nontoxic dosages of some, but not all, chemopreventive agents reduces the frequency of spontaneous immortalization.

MATERIALS AND METHODS

Materials. The chemopreventive agents nimesulide (a specific COX-2 inhibitor) and rosiglitazone maleate (a PPARγ agonist) were received from the DCP Repository (McKesson BioServices, Rockville, MD). NS-398 (a specific COX-2 inhibitor) was purchased from Cayman Chemical, and celecoxib (a specific COX-2 inhibitor) was purchased from LKT Laboratories. Pfizer (Dr. Farzan Rastinejad) kindly provided the p53 rescue drug CP-31398. Sulindac sulfide (a nonselective COX-1 and COX-2 inhibitor) was a gift from Merck Research Laboratories. The drugs were dissolved in DMSO as stock solutions with the exception of rosiglitazone maleate, which was dissolved in ethanol. The final concentrations of solvents were no >0.1%.

Cell Culture. The HMECs used for these experiments were derived from a 31-year-old LFS patient's noncancerous breast tissue [containing a germ-line mutation at codon 133 in one of the two alleles of the *p53* gene (Met to Thr [M133T]) that affects wild-type p53 protein conformation] and grown under serum-free conditions as described previously (21, 22). These cells undergo crisis around PD level 50–60 (21, 22). As is the case for other mammary epithelial cells grown under these conditions, the cells had undergone a "self-selection stage" during which the culture was overgrown by cells that lacked p16 expression, probably attributable to methylation (23, 24).

Determination of the Frequency of Immortalization. The frequency of immortalization was estimated using a fluctuation analysis as described previously (21, 22, 25, 26), in which cells were maintained in 10 different series for the last 10 doublings of their life span. Immortalization was expressed as the number of immortal cell lines obtained per number of culture series.

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[3] The abbreviations used are: LFS, Li-Fraumeni syndrome; HMEC, human mammary epithelial cell; PD, population doubling; NSAID, nonsteroidal anti-inflammatory drug; TZD, thiazolidinedione; PBS-T, PBS plus 0.05% Tween; COX, cyclooxengenase; PPAR, peroxisome proliferator-activated receptor.

Frequency was expressed as the probability of obtaining an immortal cell line based on the total number of cells plated at each passage (not per cell division) and is calculated by taking the total number of independent immortalization events (series yielding immortal cell lines) and dividing by the total number of cells plated (the number of series times the number seeded per dish).

Statistical Analysis. The data were collected as three different experiments for the cells treated with chemopreventive agents (Table 1). A two-tailed Fisher's exact test was performed to examine the association between treatment and experiment to determine whether the data across the three experiments for each treatment could be combined in additional analyses. Because there was no statistically significant association between experiment and treatment for the chemopreventive agents ($P > 0.99$), the data for each treatment across three experiments were combined. Comparisons of immortalization events for the nine chemopreventive treatments to either of the two controls (untreated or solvent) were performed using two-sided χ^2 tests of independence. When significant, these were followed with Tukey-type *post hoc* multiple comparison tests for proportions (27) to examine which treatments were statistically and significantly different. The comparisons of interest were each of the chemopreventive treatments *versus* either the untreated or solvent controls. Statistical analyses were not performed on the average frequencies of immortalization (Table 1). The α level for all statistical tests was set to 0.05, and the Fisher exact and χ^2 analyses were performed using the SAS program (Version 8.2; SAS Institute, Cary, NC). The Tukey-type *post hoc* multiple comparisons tests for proportions were programmed in Microsoft Excel, and significance was determined by consulting "Critical Values of the q Distribution" by Harter (reprinted in Ref. 27), where exact *P*s are not available.

Western Analysis. Whole cell lysates were prepared from logarithmically growing cells by lysis using 2% SDS in 50 mM Tris-HCl. Total protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) according to manufacturer's instructions. Samples (50–100 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore Corp., Marlborough, MA). The blots were washed in PBS-T, then blocked in PBS-T plus 5% powdered milk for 1 h. Monoclonal antibodies to actin (Santa Cruz Biotechnology, Santa Cruz, CA), COX-1 (Santa Cruz Biotechnology), COX-2 (Santa Cruz Biotechnology), PPARγ (a gift from David Manglesdorf, University of Texas-Southwestern Medical Center at Dallas), and p53 (Ab-6; Oncogene Science) were added to PBS-T plus milk. After washing, appropriate secondary antibodies coupled to horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom) were added. After washing, specific protein bands were detected using the chemiluminescent substrate, enhanced chemiluminescence (Amersham), and exposed to X-ray films.

Transient Transfection and Luciferase Reporter Assay to Measure PPARγ Activation. The reporter plasmids, pPPRE$_3$-TK-LUC (luciferase gene under the control of the herpes simplex virus thymidine kinase promoter), and three PPARγ response elements used in this assay have been described (28). A 250-ng measurement of pCMX-β-GAL (β galactosidase) was used as an internal control. For each experiment, LFS-HMECs maintained in culture media without antibiotics were plated 1.5×10^5/well in Falcon six-well dishes. LFS-HMECs were transfected with the indicated DNAs by using FuGENE 6 (Roche Molecular Biochemicals), according to manufacturer's guidelines. Cells were washed, fed with culture media, treated with or without rosiglitazone maleate, and harvested 24 h later. Cell extracts were assayed for luciferase activity, using the Promega luciferase assay system, and values were normalized with β galactosidase. Triplicate samples were measured in each experiment. Data are presented as fold increase over control ± SD of two experiments.

Terminal Restriction Fragment Assay. Measurements of telomere lengths were performed as described previously (29). Briefly, HMEC samples were lysed, and the proteins were digested in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 100 mM EDTA (pH 8.0), 1% Triton X-100, and 2 mg/ml proteinase K for 2 h at 55°C followed by inactivation of proteinase K for 30 min at 70°C and dialysis in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0) at 4°C overnight. Genomic DNA was digested to completion with multiple restriction enzyme mix (~1 unit/μg *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Msp*I, and *Rsa*I; Boehringer Mannheim). The digested DNA was separated on a 0.7% agarose gel in 1× TAE buffer [0.04 M Tris-acetate and 0.002 M EDTA (pH 7.6)]. The gel was denatured for 20 min in 0.5 M NaOH and 1.5 M NaCl, rinsed with distilled water for 10 min, dried on Whatman 3MM paper under vacuum for 1 h at 55°C, and neutralized for 15 min in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0). The gel was probed with a radiolabeled telomeric $(CCCTAA)_4$ probe for 16 h at 42°C in 5× SSC buffer, 5 × Denhardt's solution, 10 mM Na_2HPO_4, and 1 mM $Na_2H_2P_2O_7$. The gel was then washed twice at room temperature for 15 min each in 0.1× SSC and exposed to a phosphor screen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA).

RESULTS

Final Concentrations of the Agents Used Do Not Induce Toxicity. Initial studies tested a range (1 nM to 100 μM) of chemopreventive agent concentrations. Fig. 1 shows the dose response of LFS-derived breast epithelial cells treated 72 h with 1 nM to 100 μM of specific COX-2 inhibitors (celecoxib, nimesulide, and NS-398), a nonselective COX-1 and COX-2 inhibitor (sulindac sulfide), and the PPARγ agonist rosiglitazone maleate, compared with untreated control cells. Final concentrations (10–100 nM) of the chemopreventive agents used in these studies did not affect normal growth rates or induce toxic effects on the long-term culture of the preimmortal cells. Final concentration of CP-31398 (2 μg/ml) used in the experiments also did not affect normal growth rates (data not shown).

Table 1 *Effects on the spontaneous immortalization of LFS-derived breast epithelial cells by chemopreventive agents*

Treatment (concentration)	Immortalization events[a,b]			P[c]	Ave. frequency[d]
	Exp. 1	Exp. 2	Exp. 3		
Untreated	5/10	6/10	5/10		5.33×10^{-7}
Solvent	5/10	5/10	5/10		5.0×10^{-7}
Celecoxib (10 nM)	3/10	3/10	3/10		3.0×10^{-7}
Celecoxib (100 nM)	2/10	2/10	3/10		2.33×10^{-7}
Nimesulide (10 nM)	4/10	4/10	4/10		4.0×10^{-7}
Nimesulide (100 nM)	2/10	3/10	4/10		3.0×10^{-7}
NS-398 (10 nM)	3/10	4/10	3/10		3.33×10^{-7}
NS-398 (100 nM)	3/10	3/10	3/10		3.0×10^{-7}
Sulindac sulfide (100 nM)	5/10	5/10	5/10		5.0×10^{-7}
Rosiglitazone maleate (10 nM)	2/10	1/10	1/10	<0.05	1.33×10^{-7}
CP-31398 (2 μg/ml)	0/10	1/10	0/10	<0.05	0.33×10^{-7}

[a] Cells were treated with nontoxic concentrations of chemopreventive agents (see "Materials and Methods"). Three separate experiments (Exp.) were conducted, and the numbers of immortalization events are shown for each experiment. Immortalization events are expressed as the number of immortal events per number of culture dishes used (in this case, 10 dishes). Each dish was maintained at 10^6 cells/dish.

[b] The combined data had a χ^2 of 33.19, 11 degrees of freedom, and $P > 0.99$.

[c] *P*s from the Harter tables (reprinted in Ref. 27) from Tukey-type multiple comparison tests for proportions comparing untreated (rosiglitazone maleate and CP-31398) or solvent-treated (CP-31398) control cells.

[d] The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated, *e.g.*, if one maintained 10 series at a minimum population size of 10^6 cells/dish, for a total pool size of 10×10^6 (or 10^7), and cells in three of the series became immortal, this would yield a frequency of 3 divided by 10^7, 3×10^{-7} (Refs. 21 and 22). The average (Ave.) frequency of the three experiments is shown.

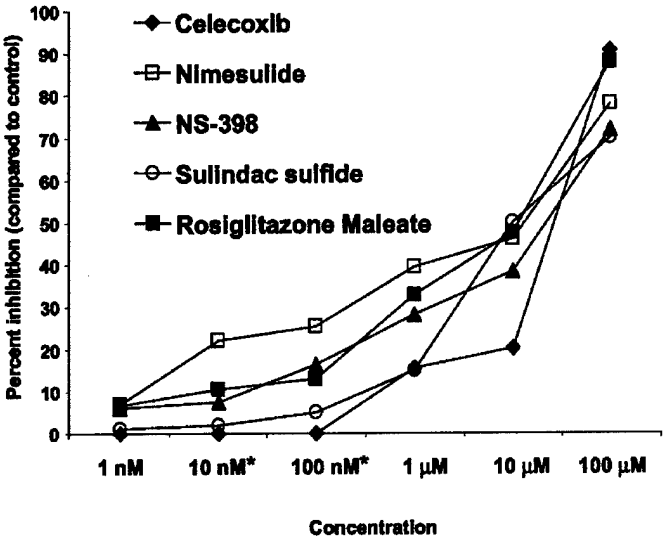

Fig. 1. Dose response of growth inhibition by chemopreventive agents. Concentrations of chemopreventive agents used for the studies (indicated by *asterisks*) were chosen to be below the levels that affected cell proliferation and were not toxic to the cells. Data indicate inhibition percentage of LFS-HMEC growth treated with the NSAIDs celecoxib, nimesulide, NS-398, sulindac sulfide, and PPARγ agonist rosiglitazone maleate for 72 h as compared with control cells.

Effect on the Spontaneous Immortalization of LFS-derived Breast Epithelial Cells by Chemopreventive Agents. Treatment with some, but not all, chemopreventive agents reduced the frequency of spontaneous immortalization of the LFS-derived breast epithelial cells. Cells were continuously treated 5–10 PDs before crisis until the fluctuation analyses ended. Table 1 shows the effects of the chemopreventive agents on the frequency of spontaneous immortalization of LFS-derived breast epithelial cells. The NSAID sulindac sulfide showed no significant effect, but there was a modest reduction in the frequency of immortalization seen with the other three COX-2-specific inhibitors (celecoxib, nimesulide, and NS-398). A frequency of $3–4 \times 10^{-7}$ compared with a frequency of $5–5.33 \times 10^{-7}$ for untreated or solvent control (Table 1) was, according to our analyses, not significant. Increasing the concentrations of the NSAIDs 10-fold to 100 nM did not significantly improve the inhibition of spontaneous immortalization (a frequency of $2.33–3 \times 10^{-7}$ compared with a frequency of $5–5.33 \times 10^{-7}$ for untreated or solvent control; Table 1). This may reflect the fact that by Western analyses, LFS-HMECs show an increase in COX-1 and a decrease in COX-2 protein levels after immortalization (Fig. 2).

Expression and Activation of PPARγ by Rosiglitazone Maleate in LFS-HMECs. The PPARγ agonist rosiglitazone maleate markedly reduced the frequency of spontaneous immortalization (1.33×10^{-7} compared with a frequency of 5.33×10^{-7} for untreated control). Western analysis with an antibody that detects both subtypes of PPARγ shows that PPARγ is present at roughly equal level in both the preimmortal and immortal LFS-HMECs, and treatment with 10 nM rosiglitazone maleate did not affect PPARγ expression levels after 24 h (Fig. 3*A*). Transient transfections with a $pPPRE_3$-TK-LUC reporter plasmid showed only minimal activation of the endogenous PPARγ after 24-h treatment with rosiglitazone maleate in the LFS-HMECs (Fig. 3*B*). This result indicates that rosiglitazone maleate may mediate its effects through a PPARγ-independent pathway in LFS-derived HMECs.

Induction of Telomere Shortening-induced DNA Damage Signals after Treatment with the p53 Rescue Drug CP-31398. CP-31398 also significantly reduced the frequency of spontaneous immortalization compared with solvent control (Table 1). Treatment with CP-31398 resulted in more cellular death late in the crisis period, compared with untreated or solvent controls. No toxicity from the treatment with CP-31398 was observed before or early in the crisis period. As shown in Fig. 4, increase of p53 and p21 protein levels (Fig. 4*A*), in addition to telomere shortening to a critical size (Fig. 4*B*), correlated with the increase in cellular death (data not shown) and inhibition of spontaneous immortalization in the LFS-HMECs during the fluctuation analyses.

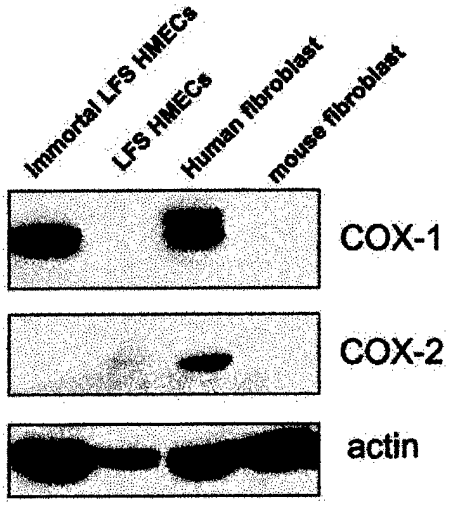

Fig. 2. Western blot analysis of COX-1 and COX-2 protein levels in LFS-HMECs and immortalized LFS-HMECs. Loading control was determined by Western blot analysis of actin. Human and mouse fibroblast lysates were included to show the species specificity of the antibody.

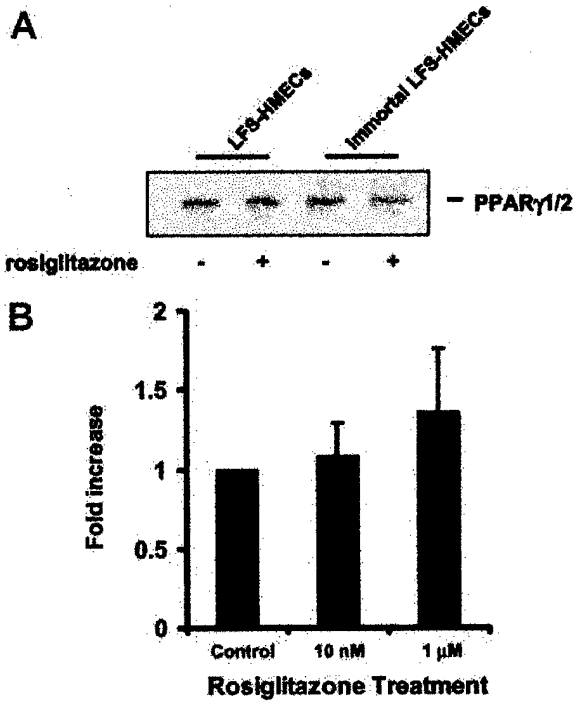

Fig. 3. Expression of PPARγ and effects by rosiglitazone maleate in LFS-HMECs. *A*, Western blot analysis of protein lysates from LFS-HMECs and immortalized LFS-HMECs without (−) or with (+) treatment of 10 nM rosiglitazone maleate for 24 h. The PPARγ antibody recognizes both PPARγ1 and PPARγ2 isoforms. Data are representative of three experiments. In *B*, LFS-HMECs were transfected with 5 ng of $pPPRE_3$-TK-LUC reporter gene. The effects of rosiglitazone maleate on the luciferase activity activated by PPARγ were measured, and data are presented as the fold increase over control (±SD) of two experiments done in triplicate. Cells received no treatment (*Control*) or treatment with 10 nM and 1 μM rosiglitazone maleate. Luciferase counts were normalized to β-galactosidase.

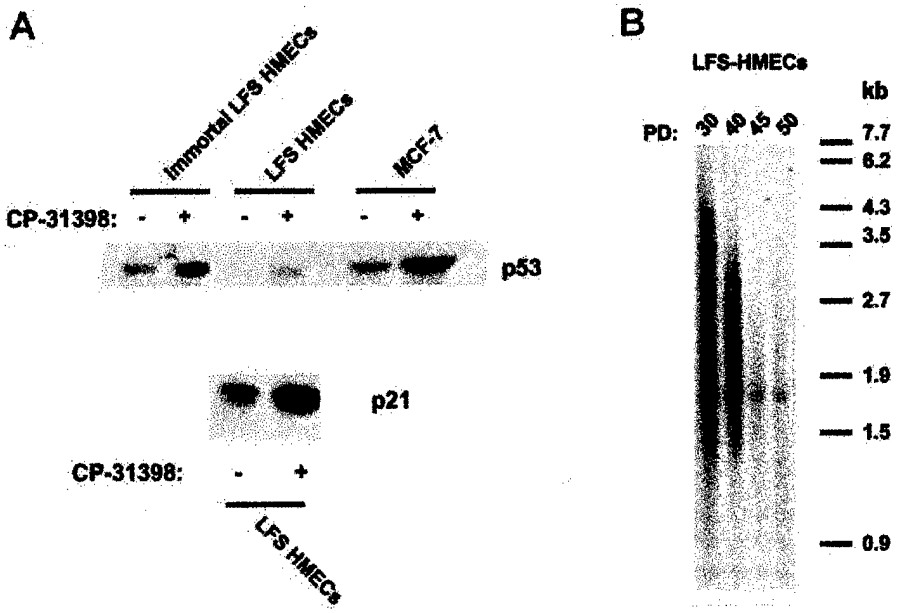

Fig. 4. Telomere shortening-induced DNA damage signals in LFS-HMECs after treatment with p53 rescue drug CP31398 correlate with inhibition of the spontaneous immortalization. *A*, Western blot analysis of p53 and p21 in treated LFS-HMECs during the fluctuation analyses, as well as analyses of p53 in treated immortalized LFS-HMECs and MCF-7 breast carcinoma cells. Data are representative of three experiments. *B*, telomere shortening to a critical length over time as evidenced by telomere restriction fragment length analyses. LFS-HMEC DNA from PD levels 30–50 were digested with six restriction fragment enzymes that do not cut within the telomeric region and probed with a telomere-specific radiolabeled probe.

DISCUSSION

It is widely accepted that chemopreventive agents may prevent or hinder the occurrence of cancer (30). Unfortunately, results from clinical trials take many years to generate. It is therefore attractive to design and test agents that act on specific molecular targets and develop preclinical models with a measurable end point to examine the effects of potential chemopreventive agents and their mechanisms of action (30). In the present study, we used an *in vitro* cell culture model system of HMECs that were derived from a patient with LFS to study the effects of several novel chemopreventive agents on the frequency of spontaneous immortalization, a measurable end point for the critical step toward cancer progression (22). We studied the effects of four NSAIDs, a peroxisome PPARγ agonist, and the P53 rescue drug CP-31398 on the frequency of spontaneous immortalization of LFS-derived breast epithelial cells.

NSAIDs block the biosynthesis of prostaglandins by inhibiting the COX activity of the enzyme prostaglandin G/H-synthase (for review, see Ref. 31). COX-1 and COX-2, two COX isoforms, are expressed in a variety of normal tissues, and COX-2 has been implicated as having a role in breast cancer as well as other cancers by stimulating cell growth through prostaglandins, suppressing apoptosis by increasing Bcl-2 and enhancing angiogenesis and cell invasiveness (reviewed in Ref. 32). The ability of NSAIDs to reduce transformation can be confined to specific oncogenic pathways, such as those involving RAS (33), which can activate COX-2 (32). Although most chemopreventive studies have been performed with prostate and colon cancer, the expressions of COX-1 and COX-2 were found to be high in human breast cancer (34). Hwang *et al.* found that COX-2 expression was particularly high in breast epithelial tumors, whereas COX-1 was primarily localized to the adjacent stromal cells, which also contained some COX-2. The fact that the NSAIDs, including specific COX-2 inhibitors (celecoxib, nimesulide, and NS-398), had only a modest effect on the frequency of spontaneous immortalization in the HMECs used in this study may be attributable to the low doses used in this study (nM range) compared with those used in anticancer studies (μM range), suggesting that the anticancer effects in culture cells are only seen at doses that are toxic for the mammary epithelial cells used in this study. More importantly, the system used in this study measures the frequency of immortalization *in vitro* and, thus, only one step in carcinogenesis. It would not address the *in vivo* chemopreventive activity of agents that affected other stages in becoming tumorigenic. The relationship between epithelial cells and their underlying stromal cells is not represented in this particular LFS-HMEC *in vitro* system. Because NSAIDs, particularity COX-2 inhibitors (such as celecoxib, nimesulide, and NS-398 used in the present study), appear to mediate their effects on stromal cells (reviewed in Ref. 35), the chemopreventive predictability of NSAIDs would be best suited by *in vivo* or other studies. Celecoxib, NS-398, and sulindac sulfone were all found to prevent mammary carcinogenesis in mice and rats (36–38). Our investigations were limited to the specific effects of potential chemopreventive agents on the immortalization frequency of LFS-derived breast epithelial cells.

Rosiglitazone maleate (rosiglitazone), a member of the TZD class of drugs, modulates the activity of the peroxisome PPARγ, a nuclear hormone receptor (39, 40). PPARγ regulates diverse physiological and pathophysiological gene expression, more specifically, cell proliferation and differentiation (39, 41). PPARγ binds to a target gene as a heterodimer with a retinoid X receptor and can act as a ligand-dependent transcription factor (39). PPARγ, normally expressed in adipose tissue, is highly expressed in tumors, and studies show reduced tumor cell growth and/or tumor cell arrest with certain PPARγ ligands, such as rosiglitazone (39, 42). Furthermore, studies of ligand-mediated activation of PPARγ in cultured breast cancer cells also show reduction in growth rate and increased differentiation and suggest chemotherapeutic applications for rosiglitazone in breast cancer treatment (42–45). Because cell differentiation and reduced cell growth may influence the immortalization process, we sought to examine rosiglitazone-mediated effects on the spontaneous immortalization frequency in breast epithelial cells from an individual predisposed to cancer (HMECs derived from an LFS patient). Our *in vitro* results, using the LFS-derived HMECs to screen for immortalization frequency, suggest the chemopreventive potential of nontoxic doses of rosiglitazone in individuals predisposed to breast cancer. However, like other members of the TZD class of drugs (ciglitizone and troglitazone), our results indicated that rosiglitazone may be working via a PPARγ-independent pathway. These TZD class PPARγ agonists were shown to sensitize a variety of cell types to the death receptor-

mediated apoptosis, further suggesting an alternative target for these ligands (46).

The high frequency of p53 mutations in cancer, especially breast cancer, makes the rescue of the tumor suppressor function an attractive mechanism for chemoprevention (47). The Pfizer compound CP-31398 has been reported to stabilize the core domain of p53 *in vitro* and be an effective anticancer drug by rescuing destabilized mutants of p53 (48). Because the breast epithelial cells derived from a patient with LFS contain an inherited mutation in p53 and lose the second functional allele when they spontaneously immortalized, we investigated if CP-31398 could rescue the mutant p53 and prevent the spontaneous immortalization of these cells. Treatment of the LFS-derived breast epithelial cells with CP-31398 significantly reduced the frequency of spontaneous immortalization. We also observed an increase in cellular death compared with untreated cells undergoing crisis, which occurred near the end of the fluctuation analyses. Other investigators have reported a nonspecific toxic effect when using CP-31398 (49). They concluded that the nonspecific effects of CP-31398 might override the p53-specific effects in cells. Our use of preimmortal HMECs in these studies suggests a different interpretation. The activation of p53 by "too short" telomeres is a key part of the DNA damage response that occurs during replicative aging. Stabilizing a mutant p53 in normal healthy cells may be nontoxic, because the p53 would be expected to lack the post-translational modifications that occur during the DNA damage response. However, as the LFS-HMECs approach their proliferation limits, the telomere shortening-induced DNA damage signals activate the stabilized p53 and produce the very late "toxic" effects we observed. This would be analogous to what has been observed in mouse studies, in which an extra copy of an activated mutant p53 produced toxicity to the tumor cells, whereas an extra copy of a wild-type p53 did not (50). Rather than being nonspecific, this would reflect a direct action mechanism of the drug. The telomere shortening in the LFS-HMECs as they reached crisis and the increase of p53/p21 protein levels seen in our studies suggest that stabilization of p53 by CP-31398 can increase telomere shortening-induced DNA damage signals, as evidenced by an increase in cellular death.

The data presented in this study provide insights into the effects of different chemopreventive agents on the spontaneous immortalization of LFS breast epithelial cells. Treatment with rosiglitazone maleate and the p53 rescue drug CP-31398 notably reduced the frequency of spontaneous immortalization of LFS-derived breast epithelial cells. The use of NSAIDs, including specific COX-2 inhibitors (celecoxib, nimesulide, and NS-398), had only a marginal effect on the spontaneous immortalization of preimmortal LFS breast epithelial cells at the concentrations tested. In summary, these studies show the usefulness of a human cellular-based model system to dissect some of the cellular pathways of known and potentially novel chemopreventive agents, using cell immortalization as an end point.

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